Uppsala 1977

SNV PM 895

Metabolism of Chlorophenols:

Studies on Dechlorination in Mammals

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Rapport från
STATENS NATURVÅRDSVERK 1977

Föreliggande rapport grundar sig på arbeten utförda med ekonomiskt stöd från forskningsnämnden vid statens naturvårdsverk.

Författaren är ensam ansvarig för rapportens innehåll, varför detta ej kan åberopas såsom representerande naturvårdsverkets ståndpunkt.

Solna i oktober 1977

Statens naturvårdsverk
Forskningssekretariatet

METABOLISM OF CHLOROPHENOLS:

STUDIES ON DECHLORINATION IN MAMMALS

by

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1977

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The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Computer-controlled mass fragmentography with digital signal processing. J. Chromatogr. 81, 47-55 (1973). Together with K. Elkin, L. Pierrou, B. Holmstedt and J.-E. Lindgren.

II. Metabolism of pentachlorophenol. Arch. Toxicol. 32, 271-281 (1974). Together with J.-E. Lindgren and M. Mercier.

III. Inhibition of β-glucuronidase by chlorinated hydroquinones and benzoquinones. Arch. Toxicol. 37, 81-87 (1977). Together with E. Manzoor and T. Thunberg.

IV. Metabolism of pentachlorophenol *in vivo* and *in vitro*. Submitted for publication in Arch. Toxicol. Together with K. Larsson and T. Thunberg.

V. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the *in vivo* and *in vitro* dechlorination of pentachlorophenol. Submitted for publication in Arch. Toxicol. Together with T. Thunberg.

VI. Metabolism of tetrachlorophenols in the rat. Submitted for publication in Arch. Toxicol. Together with K. Larsson.

ABBREVIATIONS

DDT	1,1,1-trichloro-2,3-bis-(p-chlorophenyl) ethane
DDE	1,1-dichloro-2,2-bis-(p-chlorophenyl) ethene
3-MC	3-methylcholanthrene
SKF 525-A	β-diethylaminoethyl-diphenyl propylacetate
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

INTRODUCTION

Pentachlorophenol and the lower chlorinated phenols tetra- and trichlorophenols, have been in use as fungicides, herbicides, insecticides and precursors in the synthesis of other pesticides since the early 1930's.

Production of chlorinated phenols has during the years at times been the cause of problems with regard to industrial hygiene but has otherwise essentially not created environmental problems. The introduction of modern analytical techniques, however, has revealed the ubiquitous occurrence of pentachlorophenol in the environment and the discovery of chlorinated dimers such as dibenzo-p-dioxins and dibenzofurans as impurities in commercial chlorophenol formulations have made a re-evaluation of the chlorinated phenols neccessary.

This paper summarizes studies dealing with the development of analytical methods for chlorophenols, the process of dechlorination of chlorophenols in mammals as well as some aspects of the effects of chlorinated phenols and their metabolites. Finally, it presents an effort to evaluate the hazards involved in the use of the most important chlorinated phenol, pentachlorophenol, with regard to toxicity, contaminants and degradation in the environment.

Historical background

Pentachlorophenol was synthetized for the first time by Erdmann (1841) during his studies of indigo and simultaneously by Laurent (1841). Neither of them, however, determined the correct structure of the compound. Several different methods for synthesis were later tried but the preparation of pentachlorophenol by direct chlorination of phenol in the presence of a catalyst was then reported by Merz and Weith (1872).

Modern production of pentachlorophenol or lower chlorinated phenols may be performed in two ways (Melnikov, 1971): By direct chlorination of phenol, chlorophenols or polychlorophenols with chlorine in the presence of catalysts at an elevated temperature or by alkaline hydrolysis of hexachlorobenzene or other chlorbenzenes in methanol, ethylene glycol or mixtures of different solvents.

Both methods may give rise to the formation of contaminants such as chlorinated dibenzodioxins, dibenzofurans and 2-phenoxyphenols (predioxins). According to Nilsson (1977) the most important chlorophenols produced by direct chlorination are 2,4--dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol and by alkaline hydrolysis mainly 2,4,5--trichlorophenol and pentachlorophenol. In the tragic accident in Seveso, Italy, 1976, 2,4,5-trichlorophenol was produced by alkaline hydrolysis of tetrachlorobenzene utilizing ethylene glycol as solvent (Erne and Borg, 1976; Ahlborg et al., 1977).

The chlorinated phenols were the subject of some interest in the years following their synthesis, e.g. Bechhold and Ehrlich (1906) studied their antiseptic and toxic properties, the toxicology was studied by Binet (1896) and Baylé (1924), but it was not until 1936 their usefulness was appreciated and production was started on a commercial scale. The rationals behind the introduction of pentachlorophenol as a preservative for wood and wood products have been reviewed by Carswell and Nason (1938) and Carswell and Hatfield (1939).

Since its introduction pentachlorophenol has gained world-wide recognition as an agent to control mold, mildew and termites on wood and it has also been used as a herbicide in pineapple and sugarcane fields (Bevenue and Beckman, 1967; Bevenue et al., 1967a). Pentachlorophenol has also found use as a molluscicide in the control of schistosomiasis (Roushdy et al., 1974).

The annual use in the U.S.A. is probably more than 20 000 tons and was estimated to more than 24 000 tons in 1974 (Arsenault, 1976). In Sweden chlorinated phenols as pesticides were introduced in the early 1950's. The main use has been as fungicides for wood protection and the impregnation of textiles though a certain insecticidal activity has probably been encountered in connection with the use on wood.

The annual consumption of chlorophenols in Sweden has been estimated to about 150 tons of which 90 per cent has been used for wood protection. The chlorophenols used are tri-, tetra- and pentachlorophenols.

One important field of application in Sweden was the use as a slime control agent in the paper mills. However, this application was shown to create environmental problems due to the disposal of contaminated water from the mills which caused fish toxicity and an accumulation of pentachlorophenol in fish close to the mills (Stark, 1969; Rudling, 1970). The use of chlorinated phenols for slime control was subsequently prohibited in December, 1970.

Surveys of chlorinated dioxins, dibenzofurans and chlorinated 2-phenoxyphenols (predioxins) in commercial chlorophenol preparations as well as pyrolysis experiments indicating that burning of chlorophenol-preserved wood may produce considerable amounts of chlorinated dioxins have lead to the withdrawal of the licensing for wood preservatives based on chlorophenols in Sweden, the prohibition coming into force 1978-01-01. The Swedish use of chlorophenol formulations, however, has been restricted to wood preservation and chlorophenols have been judged to be replaceable with other fungicides such as arsenics, fluorides and benzimidazoles.

TOXICITY OF CHLORINATED PHENOLS

Chlorinated phenols constitute a series of 19 compounds con-

sisting of mono-, di-, tri- and tetrachloroisomers and one pentachlorophenol. At room temperature they are all chrystalline solids with the exception of ortochlorophenol which is a liquid. Lower chlorinated phenols seem to be of limited use today and the modern data on their toxicity seems to be absent.

Acute toxicity

Early studies on the toxicity were performed due to an interest in them as antiseptic compounds (Bechhold and Ehrlich, 1906). The literature up to 1949 has been excellently reviewed by von Oettingen (1949). Summarizing the data of Karpow (1893), Binet (1896), Bechhold and Ehrlich (1906), Baylé (1924), Kehoe et al. (1939), Boyd et al. (1940), McGavack et al. (1941) and Deichman et al. (1942; 1943) the following can be stated with regard to the acute toxicity.

The toxic manifestations of monochlorophenols include muscular twitchings followed by general spasms, generalized tremors, weakness, staggering and finally collapse, i.e. a picture rather similar to that produced by unsubstituted phenol. The convulsant effect, typical of phenol, diminishes with an increase in chlorination and is entirely absent with pentachlorophenol.

Pentachlorophenol produces the following toxicological picture: An initial period of central depression followed by an increase in respiratory rate and volume as well as pulse rate, progressive neuromuscular weakness and at the end a rise in body temperature. Death is due to heart failure. There is a marked rigor mortis in the poisoned animals.

Many of the toxic effects of pentachlorophenol have later been explained as due to the uncoupling effects of the compound on oxidative phosphorylation (Weinbach, 1956). This effect has

also been demonstrated for other chlorophenols but the effect is decreased with a decrease in chlorination (Weinbach et al., 1965).

The conclusions that can be drawn from the earlier work reviewed by von Oettingen (1949) were confirmed in a study by Farquharson et al. (1958). A series of eleven chlorophenols were compared with regard to toxicity and biological actions. The authors concluded that with increasing chlorination there is an increase in toxicity in which the convulsant action of phenol is replaced by the signs characteristic of poisoning with dinitrophenol, a well-known uncoupler of oxidative phosphorylation. Based on determinations of the dissociation constants for the chlorinated phenols they suggested that the higher chlorinated phenols interfere with oxidative phosphorylation and that this is due to the chlorophenate ion. The convulsant action of the lower chlorinated phenols on the other hand was assumed to be associated with the undissociated molecule.

Chronic Toxicity

As will be seen from the following discussion, studies of the chronic toxicity of pentachlorophenol must take into consideration the degree of contamination by chlorinated dimers. Subacute and chronic toxicity studies performed before this knowledge had developed are thus not considered here.

Knudsen et al. (1974) determined the short term toxicity of pentachlorophenol upon oral administration to rats in a 12 week study at dose levels of 25, 50 and 200 ppm (corresponding to approximately 1.25, 2.5 and 10 mg/kg/day). The compound administered was stated to contain 200 ppm octachlorodibenzo-p-dioxin and 82 ppm pre-octachlorodibenzo-p-dioxin. Tetrachlorodibenzo-p--dioxins were not present. The occurrence of other dioxins or dibenzofurans is not mentioned. A dose-related decrease of calcium-deposits in the kidney was noted. At 50 and 200 ppm

several parameters such as liver weight and activity of microsomal liver enzymes were affected.

Schwetz et al. (1976) performed a 2 year study of oral administration of pentachlorophenol to rats. The content of chlorinated dimers in the commercial product used was 1 ppm hexa-, 6.5 ppm hepta-, 15 ppm octachlorodibenzo-p-dioxin together with 3.4 ppm hexa- and 1.8 ppm heptachlorodibenzofuran. Beside this the product contained 10 per cent tetrachlorophenol and 400 ppm of hexachlorobenzene.

There was no evidence of carcinogenicity when the compound was administered at dose levels sufficiently high to cause mild signs of toxicity (30 mg/kg/day). No effect levels for females were found to be 3 mg/kg/day and for males 10 mg/kg/day.

The data indicate a higher no effect level than the one found by Knudsen et al. (1974) in their 12 week study. The discrepancy may be attributable to the occurrence of chlorinated dimers not analyzed in the pentachlorophenol used by Knudsen et al. (1974).

In an 8 month feeding study (at dose levels 20, 100 and 500 ppm, corresponding to approximately 1, 5 and 25 mg/kg/day) on female rats, Goldstein et al. (1977) investigated the hepatic effects of technical and pure grade pentachlorophenol. The technical product contained 8 ppm hexa-, 520 ppm hepta-, 1.380 ppm octachlorodibenzo-dioxin and 4 ppm tetra-, 42 ppm penta-, 90 ppm hexa-, 1.500 ppm hepta- and 200 ppm of octachlorodibenzofurans. The pure pentachlorophenol contained less than 0.1 ppm of these dimers. Decreased body weight gain, a slight increase in liver glucuronyl transferase activity at the 500 ppm dose level and a pigmentation of the liver that could not be explained, were the only effects that could not be attributed to the contaminants occurring in the technical grade pentachlorophenol. The technical product produced hepatic porphyria and increased hepatic aryl

hydrocarbon hydroxylase activity, glucuronyl transferase activity, liver weight, cytochrome P-450 and microsomal heme. The effects were evident already at the dose level of 20 ppm and they are beyond doubt due to the contaminants.

Similar work, but utilizing other parameters, has been reported by Johnson et al. (1973) who found the chlorinated dimers responsible for several toxic actions of the commercial grade pentachlorophenol.

On the developing rat embryo and fetus, however, Schwetz et al. (1974) found a slightly higher toxicity of purified pentachlorophenol while pentachlorophenol (analytical grade) was found not to be teratogenic in rats (Courtney et al., 1976).

Inhalation toxicity

The acute toxicity of sodium pentachlorophenate, inhaled as an aerosol by rats, was found to be LD_{50} 11.7 mg/kg (Hoben et al., 1976a) which is considerably lower than the corresponding oral LD_{50} which has been reported to be 210.6 mg/kg (Deichmann et al., 1942). In a subsequent study by Hoben et al. (1976b) it was reported that daily respiratory exposure to pentachlorophenol did not result in an increased body burden in the rat. Daily exposures were performed from 2 to 5 days.

DISCUSSION OF EXPERIMENTAL DATA

Analysis

Due to the problems involved in the analysis of pentachlorophenol and metabolites in urine and other material (i.e. bad separation and interference from other compounds), mass fragmentography has been chosen for the analytical work in the present investigations. In mass spectrometry, a given compound gives rise to a unique fragmentation pattern, both with respect to the masses of the

various fragments and their abundance (intensity) ratios. These facts represent the basis for the technique called mass fragmentography or multiple ion detection (MID). Mass fragmentography has been defined as "the exclusive use of the spectrometer for the mass specific recording of preselected ions. A mass fragmentogram is obtained by on-line processing of data, and requires that the mass spectrometer be sequentially focused on the masses at which the ion abundance is to be measured. The sensitivity is down to picograms" (Hammar et al., 1968). The use of a mass spectrometer as a selective gas chromatographic detector thus permits that multiple ion species can be detected simultaneously with great sensitivity. The technique was first used by Sweeley et al. (1966) and further technical developments were then reported by Hammar and Hessling (1971), Klein et al. (1971, 1972), Frew and Isenhour (1972) and Kelly (1972). The development of mass fragmentography has been described in detail by Holmstedt and Palmér (1973) and Falkner (1977).

In order to facilitate the analysis we developed a computerized mass fragmentography system which is described in paper I. In that system the control of both the mass spectrometer and the signal processing is confined to the computer. The computer focuses on predetermined masses by adding to the accelerating voltage of the mass spectrometer a voltage from a digital to analogue converter and booster amplifier. The following digital treatment of the signals gives better control of baseline and gain while simultaneously allowing expansion of the channel numbers without hardware modifications. The system was developed on an LKB 9000 combined gas chromatograph - mass spectrometer which was interfaced to a PDP-12 computer with 8 K of core memory, real time clock and a digital plotter. The data and the calculated results are displayed on the computer's oscilloscope and can be presented in hard copy on the teletype or the plotter.

Almost simultaneously several groups reported the development of computer controlled techniques both for magnetic and quadropole

mass spectrometers (Holland et al., 1973; Holmes et al., 1973; Watson et al., 1973). A list of references covering the literature up to the beginning of 1975 has been compiled by Palmér and Holmstedt (1975). Recent reviews concerning the application of mass fragmentography with regard to pesticides and industrial chemicals are those of Thornburg (1977) and Holmstedt et al. (1977).

Most of the data given in paper II, IV, V and VI are based on analysis performed with the system described in paper I, though some confirmative work has been performed on an entirely new system developed on an LKB 2091 combined gas chromatograph - mass spectrometer interfaced to a computer network consisting of a PDP 11/45 with 80 K of core memory and 3 disc memories with a capacity of 2,4 Mb each and a GT/40 with 8 K of core memory.

The analysis of the chlorophenols and their metabolites have been performed after derivatization with either N,O-bis(tri--methylsilyl)acetamide (BSA) to obtain trimethylsilylethers or with diazoethane to obtain ethyl ethers of the corresponding phenols. Standard curves have been prepared for all determinations and internal standards have been used as described in respective papers. Mass spectrometric and gas chromatographic data on the tetrachlorophenols and the metabolites are given in Table 1 and 2 in paper VI and mass spectra for compounds relevant with regard to pentachlorophenol metabolism are found in Ahlborg (1977).

Especially the ethyl ethers are very well suited for mass fragmentographic analysis on an instrument with electron impact ionisation due to the suitable fragmentation with few fragments which means an improved sensitivity.

Distribution and elimination

In connection with the early experiments using ^{14}C-labelled

pentachlorophenol (paper II) rats were dosed with the compound through the drinking water in concentrations of 5 or 40 ppm during a 4-week period. The feeding was terminated and the levels of radioactivity determined in different organs (Fig. 1). The highest levels were found in liver and kidneys and the levels were related to the dose. One week after administration had been terminated, repeated determinations were performed. Measurable levels could then only be detected in liver and kidneys (Unpublished data).

The data are in good agreement with those of Larsen et al. (1972) who studied the tissue distribution of radioactivity 40 hours after a single administration of ^{14}C-labelled pentachlorophenol to rats. Jacobson and Yllner (1971) reported similar results upon autoradiographic studies in mice.

These data, together with those reported in paper II, indicate that at least most of the pentachlorophenol given is fairly rapidly eliminated from the body. This is further supported by studies on the pharmacokinetics of pentachlorophenol. In the rat 90 per cent of a single dose is eliminated within 3 days while the remaining part is eliminated more slowly, though less than 1 per cent remained after day 9 (Braun et al., 1977). Half-life values for the clearance from plasma in the magnitude of 20 hours for rats and 80 hours for monkeys have been reported (Braun and Sauerhoff, 1976; Braun et al., 1977). The species differences may be explained by the absence of metabolism in monkeys that excrete pentachlorophenol essentially unmetabolized and unconjugated and show an extensive enterohepatic circulation, as indicated by Braun and Sauerhoff (1976).

Pentachlorophenol has been shown to bind to albumin (Weinbach and Garbus, 1966) and to human and rat plasma (Hoben et al., 1976c). Hoben et al. (1976c) demonstrated that the binding capacity of human plasma was much higher than that of rat

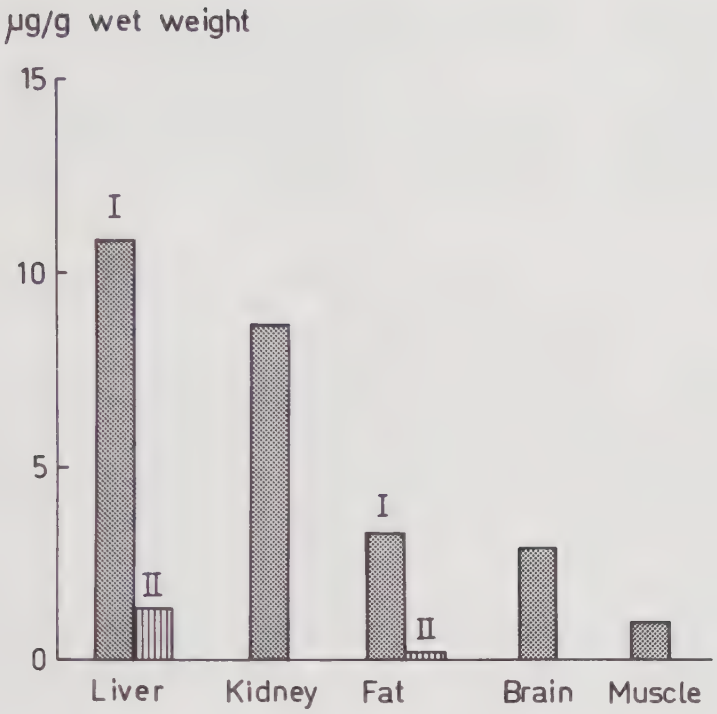

Fig. 1. Levels of ^{14}C-labelled pentachlorophenol and metabolites in different tissues after 28 days administration of ^{14}C-labelled pentachlorophenol, 40 ppm, in the drinking water to rats.

I. Immediately after cessation of administration.

II. 7 days after the administration had been terminated.

plasma, a condition that can explain the longer retention times of pentachlorophenol noted in man as compared to rats (Casarett et al., 1969). The protein binding of pentachlorophenol thus seems to explain the slow excretion of the small part of the given dose that remains after the initial rapid elimination.

Metabolism

The first study of the fate of pentachlorophenol in the body was conducted by Deichmann et al. (1942) which, contrary to what has been erroneously cited in paper II, during the first 24 hours after administration found 9% metabolism in the rabbit and 40% in the rat. The break down products were not identified and the authors concluded that conjugation with sulfuric or glucuronic acid did not take place though traces of conjugated pentachlorophenol was reported to have been found. Betts et al. (1955) found that pentachlorophenol was excreted essentially unchanged and unconjugated from the body.

In our first preliminary experiments, however, it was found that when pentachlorophenol-^{14}C was administered intraperitoneally to rats, only about 40% of the radioactivity could be recovered after adjustment of the urine to pH 2 and extraction with diethyl ether. Several other methods for extraction of pentachlorophenol (Bevenue et al., 1966; Rudling, 1970; Rivers, 1972) were tested but gave approximately the same recovery. By contrast, when pentachlorophenol was added to urine from nonexposed rats the recoveries after these extractions were almost complete.

At that time, Jacobson and Yllner (1971) reported that, after the administration of pentachlorophenol-^{14}C to mice, the compound was excreted both unchanged and conjugated and unconjugated tetrachloro-p-hydroquinone was identified as one excretion product.

In our early efforts to identify urinary excretion products thin layer radiochromatography was used (II). Upon radio-scanning of the plates after development in a solvent system with methyl acetate-isopropanol-concentrated ammonia (9:7:4) three distinct peaks occurred. The first peak was consistent with tetrachloro--p-hydroquinone and the last one with pentachlorophenol.

The second peak was believed to represent a conjugate but treatment of the urine with bacterial β-glucuronidase, aryl sulfatase or concentrated hydrochloric acid at room temperature failed to change the distribution of radioactivity. However, the second peak was absent in chromatograms of urine treated with concentrated hydrochloric acid at 100°C. Quantitatively the result was an increase of the activity of the other two peaks, the increase being more pronounced for the first peak.

The findings from the thin layer chromatographic studies were subsequently verified by gas chromatography - mass spectrometry (II).

There seemed to be good reason to believe that both pentachlorophenol and tetrachloro-p-hydroquinone were excreted partly as conjugates with glucuronic acid and the failure to split the conjugates by means of treatment with bacterial β-glucuronidase was rather puzzling. However, in experiments with bacterial β-glucuronidase using phenolphtalein-β-glucuronide or p-nitrophenyl-β-D-glucuronide as substrate, it could be shown that tetrachloro-p-hydroquinone was a potent inhibitor of bacterial β-glucuronidase activity (II).

A subsequent study (III) demonstrated that several chlorinated hydroquinones and benzoquinones were potent inhibitors of β-glucuronidase of bacterial origin with I_{50}-values in the range 10^{-6} - 10^{-7} M. These concentrations are far below those occurring in the urine from the pentachlorophenol-treated rats and the failure of bacterial β-glucuronidase to split possible glucuro-

nides is thus explained. The inhibition was found to be competitive in nature. No inhibitory effect of the benzo- and hydroquinones studies *in vitro* or *in vivo* could be demonstrated on β-glucuronidase from liver.

The results demonstrate the inhibitory potency with regard to β-glucuronidase activity of an entirely new class of compounds and call for precaution when using β-glucuronidase of bacterial origin in the study of metabolism.

In order to solve the question of conjugation, urine from pentachlorophenol dosed rats was treated in three different ways (IV):

a) Acidification with concentrated hydrochloric acid to pH 2.
b) Boiling with concentrated hydrochloric acid.
c) Incubation with β-glucuronidase from bovine liver and after that acidification to pH 2.

Both types of hydrolytic treatment (b and c) increased the recovery of free pentachlorophenol and tetrachloro-p-hydroquinone and the total increase was about 50%. The major part of this increase was due to a three- to four-fold increase in the recovery of tetrachloro-p-hydroquinone. This is indirect evidence for the presence of both compounds as conjugates with glucuronic acid.

Recently the presence of pentachlorophenol-glucuronide in urine from exposed rats has been reported (Braun and Sauerhoff, 1976; Braun *et al.*, 1977). The level of conjugated pentachlorophenol they found (9.4%) is in the same range as found by us (9-16%). However, they only found unconjugated tetrachloro-p-hydroquinone which is in clear contrast to our findings where the greater part of tetrachloro-p-hydroquinone occurs as a conjugate.

Dechlorination

Metabolic dechlorination has been studied for a number of compounds but no clear picture of the enzymatic mechanisms responsible seems to be available. At least three different metabolic pathways have been described (Parke, 1968), i.e. (i) dehydrochlorination which can be demonstrated with the metabolism of the insecticide DDT to DDE, (ii) hydrolytic dechlorination which has been demonstrated with the metabolism of 2,4,6--trichloroaniline which is converted to 4-amino-3,5-dichlorophenol and (iii) reductive dechlorination as demonstrated by the metabolism of carbon tetrachloride to chloroform. A recent example of demonstrated hydrolytic dechlorination is the metabolism of hexachlorobenzene to pentachlorophenol (Koss et al., 1976).

The mechanism of dechlorination of pentachlorophenol seems not to be very well known and the experiments reported in paper IV and V were performed to achieve a better understanding of this process. Rats were pretreated with phenobarbital, a well-known inducer of microsomal metabolizing enzymes, and with β-diethyl-aminoethyl--diphenyl propylacetate (SKF 525 A), a similarly well-known inhibitor of microsomal enzymes. As could be expected, phenobarbital increased the metabolic conversion of pentachlorophenol to tetrachloro-p-hydroquinone. SKF 525-A, however, produced a striking increase in the dechlorination of pentachlorophenol when given in a dose of 25 mg $\cdot$ kg^{-1} one hour before pentachlorophenol. Further experiments revealed that increasing dechlorination could be demonstrated until SKF 525-A was administered repeatedly in a dose of 50 mg $\cdot$ kg^{-1} every 6 hours for 24 hours. Following the latter treatment, however, a decrease in the metabolism was noticed during the second 24 hours though no SKF 525-A was administered during that period.

Several authors have reported a biphasic effect of SKF 525 A (Serrone and Fujimoto, 1962; Kato et al., 1964). Our data indicate that the inhibitory action of SKF 525 A as concerns dechlorination, seems to be rather shortlasting.

The data from the in vitro experiments (IV) show an increase in the metabolic conversion of pentachlorophenol to tetrachloro-p--hydroquinone when using liver microsomes from rats pretreated with phenobarbital and a pronounced inhibitory action of SKF 525 A both on the activity of control microsomes and induced microsomes.

The lack of inhibition in vivo thus seems to be due to the induction being more significant than the inhibition when summarized during the time interval studied.

Poland and Glover (1973; 1974) have shown that 2,3,7,8-tetrachloro--p-dibenzo-dioxin (TCDD) is a potent inducer of e.g. aryl hydrocarbon hydroxylase and that TCDD is more potent than any other inducer on a dose per unit body weight basis. In a comparison between TCDD and 3-methylcholanthrene (3-MC) they found that the maximal induction that can be achieved was the same for the two compounds. The effect of TCDD was, however, considerably more longlasting.

Using the same dosage of TCDD and 3-MC as did Poland and Glover (1974) the dechlorination activity in vivo and in vitro was compared after pretreatment with phenobarbital, TCDD and 3-MC.

In the in vivo experiments with rats induced with TCDD, the occurrence of trichloro-p-hydroquinone was identified as an excretion product in the urine from animals dosed with pentachlorophenol. The compound had earlier been tentatively identified in urine (II) but was at that time thought to be due to the presence of tetrachlorophenols occurring as impurities in the pentachlorophenol used. That minor amounts of trichloro-p--hydroquinone appear as a metabolite to 2,3,4,5- and 2,3,4,6--tetrachlorophenol could also be verified (VI).

Trichloro-p-hydroquinone could now be demonstrated to occur as a minor metabolite of pentachlorophenol. The pentachlorophenol used in paper V had a purity of 99.9 per cent and the fact that the amounts of trichloro-p-hydroquinone formed are related to the different inducing pretreatments can only be explained as the result of a continued dechlorination of pentachlorophenol.

The total amounts of pentachlorophenol, tetra- and trichloro-p--hydroquinone (in mg) excreted in the urine during the first 24 hours after the administration of pentachlorophenol intraperitoneally under the various pretreatment conditions are given in Fig. 1, paper V. Pretreatment with phenobarbital increased the amount of tetrachloro-p-hydroquinone excreted but had no influence on the amount of trichloro-p-hydroquinone formed. Pretreatment with 3-MC or TCDD increased the amount of tetrachloro--p-hydroquinone excreted even more, but also increased the amount of trichloro-p-hydroquinone. Pretreatment with phenobarbital did not affect the summarized excretion as compared to the control but pretreatment with 3-MC or TCDD increased the summarized excretion by about 60 per cent, essentially during the first 24 hours.

The results of the *in vitro* incubations (V), are in good agreement with those demonstrated *in vivo*, the dechlorination activity being more pronounced when using microsomes from rats pretreated with 3-MC or TCDD than when using microsomes from phenobarbital pretreated rats. In the *in vitro* experiments SKF 525 A was added to the incubations in a concentration of 10^{-4} M. The inhibitory effect was most pronounced for the incubations with microsomes from control or phenobarbital treated rats.

Studies of the metabolism of pentachlorophenol (II, IV and V) thus show that rapid dechlorination occurs in rats. The dechlorination is mediated by liver microsomal enzymes and their activity can be enhanced by pretreatment with inducing agents such as phenobarbital, 3-methylcholanthrene and TCDD. The dechlorination

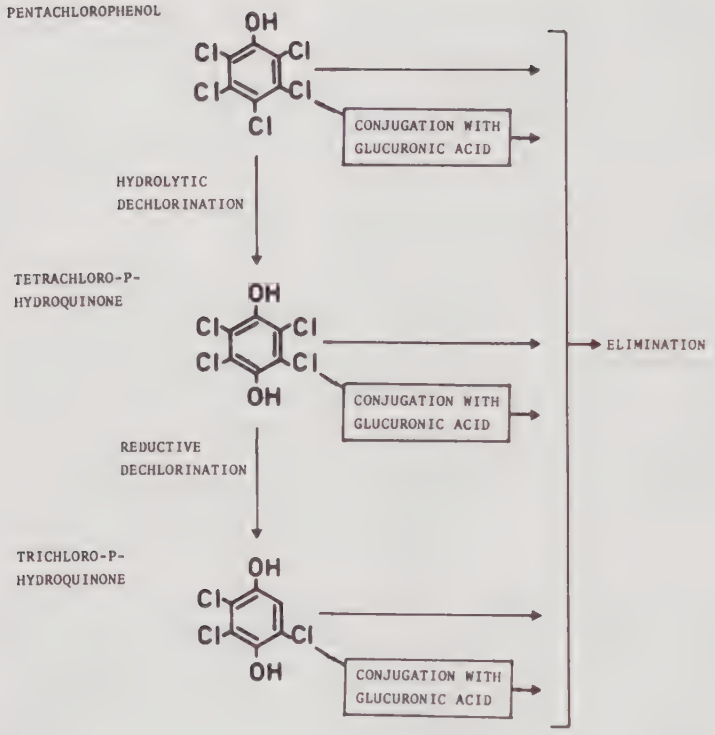

Fig. 2. Proposed scheme for the metabolic fate of pentachlorophenol in the rat.

products formed are tetrachloro-p-hydroquinone and trichloro-p--hydroquinone. The dechlorination proceeds with an initial hydrolytic dechlorination to tetrachloro-p-hydroquinone followed by a reductive dechlorination to trichloro-p-hydroquinone (Fig. 2).

Other reports have indicated the presence of dechlorination products such as tetrachloro- and trichlorophenols (Engst et al., 1976) and tetrachloro-p-benzoquinone (Tashiro et al., 1970). In our studies, these findings have not been verified.

Metabolism and elimination of tetrachlorophenols

Of the three isomers, only 2,3,5,6-tetrachlorophenol is metabolized to a significant degree (paper VI), urinary excretion of tetrachloro-p-hydroquinone constituting about 35 per cent of given dose. Trichloro-p-hydroquinone was identified as a minor metabolite of both 2,3,4,5- and 2,3,4,6-tetrachlorophenol. Elimination was rapid for 2,3,5,6- and 2,3,4,6-tetrachlorophenol, while only 60 per cent of the given dose could be recovered in the urine within 72 hours after administration of 2,3,4,5-tetrachlorophenol. The toxicity was lower than for pentachlorophenol but varied considerably between the isomers, the oral LD_{50}'s being 109, 131 and 400 mg/kg body weight for 2,3,5,6-, 2,3,4,6- and 2,3,4,5-tetrachlorophenol respectively.

The levels of chlorinated dimers in the different isomers have been analyzed (Jansson, 1977) and 2,3,4,5- and 2,3,5,6-tetrachlorophenol contained less than 1 ppm of any dibenzo-p-dioxins with 4, 5, 6, 7 or 8 chlorine. 2,3,4,6-tetrachlorophenol contained 5, 40 and 33 ppm of dibenzo-p-dioxins with 6, 7 and 8 chlorine respectively. The latter compound, however, was subjected to the purification procedure described in paper VI, before experimental use, and it is likely that this procedure would decrease the level of dimeric contaminants as well, though no analysis for dimers was performed after purification. Due to the low levels present it is thus unlikely that the contaminants in any way would have influenced the result of either the studies of metabolism or determinations of acute toxicity.

Toxicity and effects of tetrachloro-p-hydroquinone

Data on the toxicity of tetrachloro-p-hydroquinone have not been found in the literature with the exception of a report by Staub (1926), who, in a study of possible contaminants in commercial tetrachlorobenzoquinone (chloranil), could not show liver damage in rabbits. After giving oral dosages up to 900 mg · kg^{-1} of trichloro-p-hydroquinone, the result was also negative.

In our determinations of the acute toxicity (paper VI), tetrachloro-p-hydroquinone could be demonstrated to have a low oral toxicity in mice when compared to pentachlorophenol (LD_{50} 500 mg · kg^{-1} and 74 mg · kg^{-1}, respectively). On the other hand, after intraperitoneal administration, the test did not reveal any difference between pentachlorophenol and its main metabolite, tetrachloro-p-hydroquinone. The difference in toxicity, after oral or intraperitoneal administration of the latter compound, may be explained by a possibly rapid oxidation to tetrachlorobenzoquinone in the intestine.

The effect of pentachlorophenol on oxidative phosphorylation (Weinbach, 1956) has been demonstrated to be related to the increase in body temperature (Buffa et al., 1963) that was noted in pentachlorophenol intoxications already by Baylé (1924).

This was taken advantage of in order to test for a similar effect of tetrachloro-p-hydroquinone. In experiments on rats it could be demonstrated (Unpublished data) that pentachlorophenol, when given at elevated room temperature (31°C), caused hyperthermia, no such effect could be seen after the administration of tetrachloro-p-hydroquinone. At an environmental temperature of 25°C the rats ability to regulate body temperature is apparently sufficient, and pentachlorophenol does not induce hyperthermia, while tetrachloro-p-hydroquinone under these conditions causes a significant

hypothermia (Fig. 3, I and II). From these data it seems likely that tetrachloro-p-hydroquinone does not possess uncoupling activity.

In studies of the metabolism of tetrachloro-p-hydroquinone, trichloro-p-hydroquinone has been identified as an urinary metabolite, constituting about 3% of the dose given, all as conjugate (unpublished data). 13% of the tetrachloro-p-hydroquinone occurred in the urine as conjugate in rats with no pretreatment. Pretreatment with TCDD, as described in paper V, did not significantly increase the amount of trichloro-p-hydroquinone formed but increased the amount of conjugated tetrachloro-p-hydroquinone to about 50%. The low amount of trichloro-p-hydroquinone formed is probably explained by the rapid elimination of the parent compound, almost 100% being eliminated in the urine during the first 24 hours.

Both tetrachloro-p-hydroquinone (paper II and III) and trichloro--p-hydroquinone (paper III) have been demonstrated to be potent inhibitors of bacterial β-glucuronidase, the I_{50}'s being $4.7 \cdot 10^{-7}$ M and $6.5 \cdot 10^{-7}$ M, respectively.

EVALUATION OF HAZARDS OF PENTACHLOROPHENOL

In an evaluation of the environmental hazards of the use and occurrence of pentachlorophenol, in addition to the toxicity of the compound itself there are three issues that have to be evaluated separately, i.e. (i) the occurrence and sources of pentachlorophenol, (ii) the impact of the impurities in technical grade pentachlorophenol and finally (iii) the possible degradation products of pentachlorophenol in the environment.

Occurrence and sources

The occurrence of pentachlorophenol in water, soil and sewage

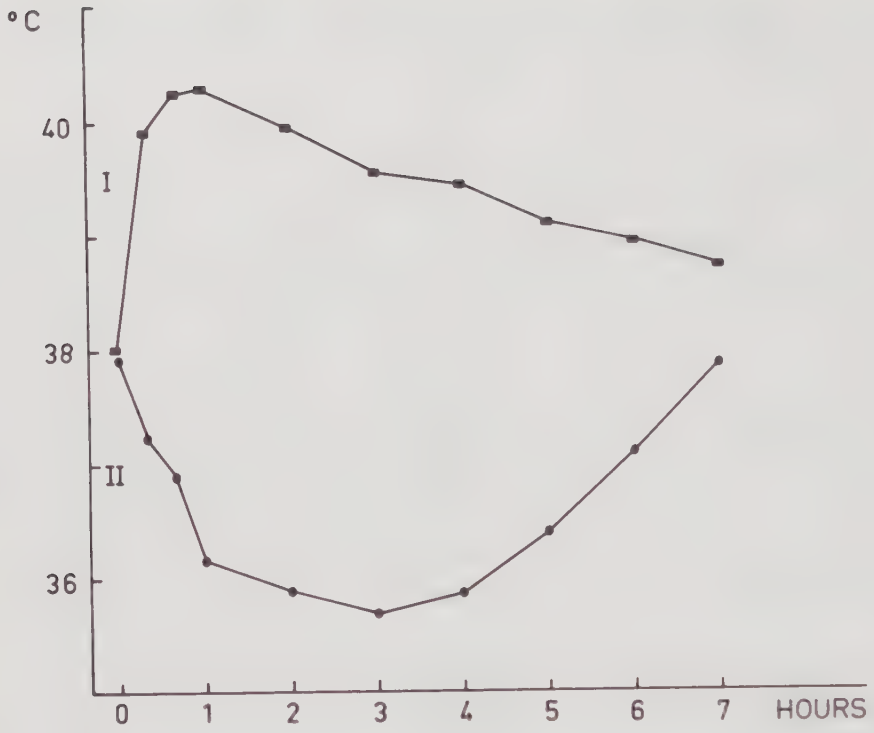

Fig. 3. Rectal body temperature in male Sprague-Dawley rats.

I. After intraperitoneal administration of pentachlorophenol 20 mg · kg^{-1}. Environmental temperature 31°C. The data differ significantly from controls during the time interval 1-5 hours ($p<0.05$).

II. After intraperitoneal administration of tetrachloro-p-hydroquinone, 20 mg · kg^{-1}. Environmental temperature 25°C. The data differ significantly from controls during the time interval 1-5 hours ($p<0.05$).

Each data represents the mean of 8 rats. Compounds were administrated in propylene glycol (2 ml · kg^{-1}). Controls were given only solvent and did not differ significantly from rats given tetrachloro-p-hydroquinone 20 mg · kg^{-1} under conditions as in I, nor from rats given pentachlorophenol 20 mg · kg^{-1} under conditions as in II.

has been demonstrated by Stark (1969), Rudling (1970) and Buhler et al. (1973) and in fish by Stark (1969) and Rudling (1970). Using negative chemical ionization mass spectrometry, Dougherty et al. (1975; 1977), demonstrated the presence of low levels of pentachlorophenol in a variety of food.

Several surveys have been done on the occurrence of pentachlorophenol in the urine, both of exposed workers and nonoccupationally exposed persons (Bevenue et al., 1967b; Cranmer and Freal, 1970; Akisada, 1965; Dougherty et al., 1975; Wyllie et al., 1975; Arsenault, 1976). Pentachlorophenol could be found virtually in all the samples analyzed, the levels varying from 0.003 ppm to almost 10 ppm in urine from heavily exposed workers. If all the data from non-exposed persons are correct it would implicate that pentachlorophenol is quite ubiquitous in the environment. It is, however, not unlikely that interference from other compounds may have influenced some analyses. Interestingly enough, chlorinated phenols may be produced in nature as indicated by Anchel (1952) who identified an antibiotic compound, Drosophilin A, as p-methoxy--tetrachlorophenol. Drosophilin A is a metabolite of a fungus utilizing potassium chloride as the only source of chlorine.

The pentachlorophenol measured in human urine may, however, partly be the result of exposure to other pesticides in the environment. Pentachlorophenol has been shown to be a metabolite of hexa- and pentachlorobenzene in rat, mouse, guinea pig, laying hen and rainbow trout (Lui and Sweeney, 1975; Koss et al., 1976; Koss and Koransky, 1977; Kohli et al., 1976). Koss et al., (1976) also identified tetrachloro-p-hydroquinone as a metabolite of hexachlorobenzene in the rat and this compound might easily be measured as pentachlorophenol when performing analysis by means of gas chromatography. Experiments with model ecosystem have demonstrated that hexachlorobenzene is slowly converted to pentachlorophenol (Lu et al., 1977). Furthermore, pentachlorophenol, as well as other lower chlorinated phenols, have been

identified as urinary metabolites of lindane (γ-hexachlorocyclohexane in rats (Engst et al., 1976) and in rabbits (Karapally et al., 1973).

Other chlorinated aromatics may also occur as environmental contaminants. Chlorophenols (di- and tri-), chloroguaiacols (di-, tri- and tetra-) and chlorocatechols (di-, tri- and tetra-) have thus recently been identified in spent bleach liquors from the bleach plant of kraft pulp mills in Sweden (Lindström and Nordin, 1976).

Impact of impurities

A number of papers have reported the occurrence of chlorinated dibenzo-p-dioxins, chlorinated 2-phenoxyphenols and chlorinated dibenzofurans in technical grade pentachlorophenol (e.g. Rappe and Nilsson, 1972; Jensen and Renberg, 1972; Firestone et al., 1972; Woolson et al., 1972; Villanueva et al., 1973; 1975; Crummet and Stehl, 1973; Buser, 1975; Buser and Bosshardt, 1976; Blaser et al., 1976; Pfeiffer, 1976; Mieure et al., 1977).

There is a considerable variance in the levels of dimers reported which by all probability indicates two things - (i) the content of dimers varies between different labels of pentachlorophenol and even between different batches from the same production site and (ii) the analytical methods used may sometimes have lead to erroneous results. Depending on the work-up procedure and derivatization technique, chlorinated 2-phenoxyphenols may be interpreted as dioxins (Rappe and Nilsson, 1972; Jensen and Renberg, 1972; 1973) and due to interfering compounds the levels may be much higher when gas chromatography with electron capture detection is used than when the more accurate method of gas chromatography - mass spectrometry is used (Arsenault, 1976).

2,3,4,4,5,6-hexachloro-2,5-cyclohexadien-1-one may be formed during the chlorination of phenol to pentachlorophenol and thus occur in commercial pentachlorophenol. It has been demonstrated that this compound can interfere with the determination of octachlorodibenzo-p-dioxin (Wilkinson, 1975).

The content of dioxin impurities in pentachlorophenol is thus variable but the main dioxins, found with their usually maximal levels, are octachlorodibenzo-p-dioxin, 2.000 ppm, heptachlorodibenzo-p-dioxin, 200 ppm, and hexachlorodibenzo-p-dioxin, 100 ppm. TCDD has not been shown to occur as a contaminant of pentachlorophenol and due to the ways of synthesis it would not be expected to occur.

The levels of 2-phenoxyphenols in pentachlorophenol may sometimes supersede several thousand ppm (Jensen and Renberg, 1973; Levin et al., 1976; Ahling et al., 1977). Analyses of 2-phenoxyphenols and their role as precursors to chlorinated dibenzo-p--dioxins have recently been discussed by Nilsson (1977) and Levin et al. (1976) have also presented data that indicate an enrichment of chlorinated dimers in the waste from sawmills using wood preserved with chlorinated phenols contaminated with these dimers.

When trying to evaluate the hazards involved due to the occurrence or formation of chlorinated dimers it is essential to distinguish two facts (i) the types of chlorinated dibenzo-p-dioxins that can be formed and (ii) their toxicity.

In different studies of the in vitro induction of δ-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase in chick embryo livers with various dioxin isomers, it has been found that the effects are dependent upon degree and location of the chlorine atoms (Poland and Glover, 1973; Bradlaw et al., 1976). The acute toxicity of the chlorinated dibenzo-p-dioxins has recently been

shown to behave in a similar manner when tested on mice and guinea pigs (McConnell et al., 1976). TCDD was the most toxic compound. With fewer chlorine atoms the toxicity was diminished with a factor 1.000 to 10.000. The addition of one or two chlorine atoms to the TCDD-structure also decreased the toxicity but only with a factor less than 100. Further chlorination, however, markedly decreased the toxicity. The toxicity was also related to chlorine in positions 2, 3, 7 and 8. Pentachloropredioxins were found to have very low toxicity when compared to the dioxins.

Degradation products

Several studies of the degradation of pentachlorophenol in soil, water and different model ecosystem have been performed. Degradation seems to take place fairly rapidly with the formation of various products such as lower chlorinated phenols, chlorohydroquinones, chlorobenzoquinones and methyl ethers of penta- and tetrachlorophenol. In general, as far as this has been studied, the toxicity of the products formed is lower than that of the parent compounds. The degradation process has been reviewed by Arsenault (1976) and additional studies were presented at a recent symposium on pentachlorophenol (Rao, 1977).

An interesting example of environmental contamination with degradation products has been revealed in the broiler industry. Feeding chickens on wood shavings from wood treated with chlorophenols has been reported to induce a musty taint in chicken meat and eggs (Engel et al., 1966; Curtis et al., 1972). The cause has been established to be the occurrence of mainly 2,3,4,6-tetrachloro-anisole formed by the methylating action of certain fungi present in poultry litter (Curtis et al., 1974; Gee et al., 1974; Parr et al., 1974). Methylation of trichloro- and pentachlorophenol was also demonstrated and it is thus obvious that microorganisms can degrade chlorophenols by methylation.

The predominant issue, however, has been the possible formation of chlorinated dimers as degradation products by the action of sunlight or by burning the treated wood, as well as considerations concerning the hazards of fire in storages of pentachlorophenol.

Irradiation of solutions with high concentrations of pentachlorophenol may, under certain conditions, lead to the formation of octachlorodibenzo-p-dioxin (Plimmer et al., 1973; Stehl et al., 1973; Wong and Crosby, 1977). Much of the evidence available, however, indicates that this is only a minor degradation pathway as compared to the formation of monomers with lower chlorine content. The breakdown of the fairly lowtoxic octachlorodibenzo--p-dioxin is rather slow when compared to the breakdown of TCDD (Crosby et al., 1973).

The burning of paper or wood treated with pentachlorophenol does not indicate any formation of octachlorodibenzo-p-dioxin contrary to when burning paper treated with sodium pentachlorophenate (Stehl et al., 1973).

There also exists more recent studies of the emission of chlorinated dimers from the burning of wood treated with technical grade pentachlorophenol (Ahling et al., 1977). The formulation used contained small amounts of octa-, hepta- and hexachloro--dimers (40, 80 and 6.5 ppm, respectively) and in addition 6.000 ppm of nonachloropredioxins and 5 per cent 2,3,4,6-tetrachlorophenol (Jansson, 1977). The results of mass fragmentographic analysis indicate that almost no formation of expected dimers occurred but rather a reduction of the existing amounts. Levels of pentachlorodibenzo-p-dioxins and tetrachlorodibenzo--p-dioxins corresponding to 17.5 and 2.1 mg/kg burned pentachlorophenol were, however, noted under pyrolytic conditions, i.e. in an oxygen-deficient atmosphere. The formation of the latter dioxins is difficult to explain. Considering all the data

it is noteworthy that the overall levels are rather low especially with regard to the enormous amounts of predioxins available in the formulation burned. Furthermore, the concentration of pentachlorophenol in the burned wood was approximately 10 times higher than usually occurring in commercial wood preservation.

From the experiments of Ahling et al. (1977) it seems logical to draw the conclusion that the burning of wood, preserved with pentachlorophenol of acceptable purity, would not imply any risk of environmental contamination with chlorinated dioxins. However, as reported by the same authors, the burning of wood treated with various technical tetra- and trichlorophenol formulations may, under certain conditions, produce remarkably high amounts of tetra-, penta- and hexachlorodibenzo-p-dioxins.

In summary, pentachlorophenol seems to be widely dispersed in the environment, mostly due to the use of the compound itself but partly due to its occurrence as a degradation product of other compounds. Technical grade formulations may contain levels of chlorinated dimers of toxicological significance. Studies on the environmental degradation of pentachlorophenol do not seem to indicate any formation of compounds that significantly add to the hazards. The burning of wood treated with pure pentachlorophenol would not likely cause environmental problems.

Thus it appears that a continued use of pentachlorophenol with an acceptably low level of contaminants would not imply any hazard provided that pentachlorophenol is handled with the precautions necessitated by its own toxicity.

SUMMARY AND CONCLUSIONS

1. Pentachlorophenol is metabolized in the rat with the formation of pentachlorophenol-glucuronide, tetrachloro- and trichloro-p-hydroquinone together with their corresponding glucuronides. 2,3,5,6-tetrachlorophenol is metabolized with the formation of tetrachloro-p-hydroquinone and the corresponding glucuronide. Only traces of dechlorinated products from the other tetrachlorophenol isomers could be found.

2. The dechlorination of pentachlorophenol proceeds with a hydrolytic dechlorination to tetrachloro-p-hydroquinone which is further subjected to reductive dechlorination. The dechlorination is mediated by liver microsomal enzymes that can be induced with e.g. phenobarbital, 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-p-dioxin.

3. The metabolites tetrachloro- and trichloro-p-hydroquinone have been demonstrated to be potent inhibitors of bacterial β-glucuronidase.

4. The data available indicate that mammals easily dispose of pentachlorophenol and that the compound or its metabolites will not accumulate in the body.

5. Pentachlorophenol in itself is a compound with an acute toxicity demanding certain precautions in handling. The main hazard, from environmental and occupational points of view, seems presently to be connected with contaminants in technical grade products. The adequate use of pentachlorophenol, of an acceptable purity, would not imply any hazard with regard to environmental or occupational hygiene. However, this conclusion does not apply to tetra- and trichlorophenols, the use of which, due to possible degradation products, may cause environmental pollution of significance.

ACKNOWLEDGMENTS

This study has been carried out in the Department of Toxicology, Swedish Medical Research Council, at the Karolinska Institute, Stockholm, and the Toxicological Laboratory of the National Food Administration, Uppsala.

I want to express my feelings of gratitude to several friends:

Bo Holmstedt for teaching me toxicology and for invaluable advice and generous support.

Lennart Albanus for his support and for never failing to push.

Sven Ullberg for his interest and support.

Keith Elkin, Kerstin Larsson, Jan-Erik Lindgren, Ellu Manzoor, Michel Mercier and Tuula Thunberg for stimulating cooperation.

Eva Nilsson and Susanna Nyiredy for excellent technical assistance

Anna Lena Marcus for skill, patience and her admirable tolerance when working under stress on the manuscripts.

Jan Dich, Jan-Erik Lundberg, Gösta Lundgren, Lena Palmér and Gunnel Quensel for lots of help.

Audrey Grönquist for patiently correcting my grammatical errors.

Financial support was obtained from the National Swedish Environment Protection Board, the Wallenberg Foundation and the Tri-Centennial Fund of the Bank of Sweden.

THANKS

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Journal of Chromatography, 81 (1973) 47–55
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 6694

COMPUTER-CONTROLLED MASS FRAGMENTOGRAPHY WITH DIGITAL SIGNAL PROCESSING

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(Received March 8th, 1973)

SUMMARY

A system for multiple ion detection has been developed in which both the control of the gas chromatograph–mass spectrometer (GC–MS) and signal processing are confined to the computer. The computer focuses on a certain mass by adding to the accelerating voltage of the GC–MS a voltage from a digital to analog converter and booster amplifier.

The digital treatment of the signals offers more satisfactory control of the baseline and gain and at the same time allows expansion of the number of channels without hardware modification.

The sampled data are continually displayed on the oscilloscope of the computer and all questions and answers are observed on the oscilloscope. The operator can set a baseline level, sample rate and gain for each channel.

The data and calculated results are displayed on the oscilloscope and can be presented as hard copy on the teletype or plotter.

The usefulness of the new system is demonstrated with regard to the metabolism of pentachlorophenol.

INTRODUCTION

By using a mass spectrometer (MS) as a selective gas chromatographic (GC) detector, multiple ion species can be detected simultaneously with great sensitivity. This technique was first used by Sweeley *et al.*[1]. Further technical developments have been described both for magnetic instruments[2–7] and for quadrupoles[8–10]. All of these systems have used analog filters, different mass channels and sequence control units to select the accelerating voltages.

The development reported here concerns the total computerization of the process, where the GC–MS is controlled by the computer and the signal processing is confined to the computer and carried out digitally. An example of the usefulness of the technique is described with regard to the metabolism of pentachlorophenol in the rat.

MASS FRAGMENTOGRAPHY EQUIPMENT

An LKB 9000 combined gas chromatograph–mass spectrometer (GS–MS) was used. This instrument was interfaced to a PDP-12 computer with 8K of core memory, a real-time clock (Digital Equipment Corp.) and a digital plotter (Houston Omnigraph).

SYSTEM DESCRIPTION

Hardware

The interface hardware system consists of a voltage control (I), a baseline control (II) and a gain control (III), which are used sequentially for all channels (Fig. 1).

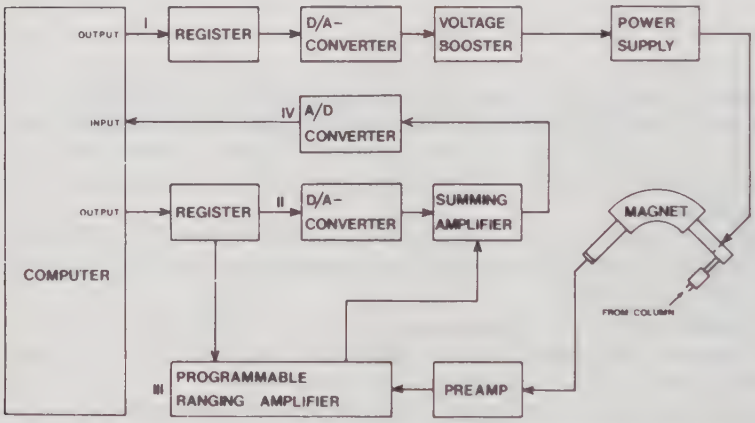

Fig. 1. Schematic diagram of computer-controlled mass fragmentography with digital signal processing.

The accelerating voltage for the mass spectrometer is controlled by the computer through a 14-bit digital-to-analog (D/A) converter and a voltage booster located in the voltage control unit (Fig. 1, I). The computer focuses on a certain mass by adding to the accelerating voltage of the mass spectrometer a voltage ranging from 0 to 1000 V that allows a mass range of 30% (calculated on the lowest mass) to be covered.

The baseline unit permits computer-controlled baseline adjustments in order to balance out any signal caused by column bleed and to bring the baselines of the different channels to the same level. This is accomplished by the addition of a d.c. voltage from an 8-bit D/A converter to the analog signal (Fig. 1, II).

The analog signal from the electron multiplier amplifier of the gas chromatograph–mass spectrometer is processed by a 6-bit programmable gain-ranging amplifier (Fig. 1, III). The use of this latter amplifier permits a more accurate determination of the selected mass area and permits a large dynamic range of the analog

mass spectrometer signal. This analog signal is converted by a 10-bit analog-to-digital converter (Fig. 1, IV) into digital information, which is stored in the computer. The accelerating voltage and the analog input parameters (gain and baseline) are simultaneously loaded into the respective control units by the software.

Software

A program that samples simultaneously a user-specified number of different masses and records the resulting data is the basis of this system. The data are continually displayed on the oscilloscope of a PDP-12 computer and all questions and answers are observed on the oscilloscope.

The system consists of the software floating-point package, the user program display routines, interface control routines and data buffers. The user parameters specify the mass, baseline level, sample rate and a gain for each channel. These parameters are accepted by the user program as described in the analytical procedure and used by the other routines for the control of the interface unit and the collection of data.

Fig. 2. Options displayed on the oscilloscope.

The operator has several options to choose from on the oscilloscope (Fig. 2):

F —FRAGMENT is used to collect data from the mass spectrometer and is followed by a request for the user parameters, as described later.

R —RUN is used to load and start a user program from the system library.

D —TO DIAL is used to return to the monitor.

P —PLOT permits a plot of the displayed data.

L —LOAD is used to retrieve previously stored data.

S —SAVE	is used to store data on the magnetic tape.
A —AUTO	is used to set a program mode to store the data automatically on magnetic tape.
C —CAL.	is used to re-calculate peak areas and peak area ratios with new retention times.
M —MODIFY	is used for background subtraction and for treatment of data.

After the specification of user parameters, the different channel curves are displayed on the oscilloscope as data are collected (Fig. 3). The data collection starts

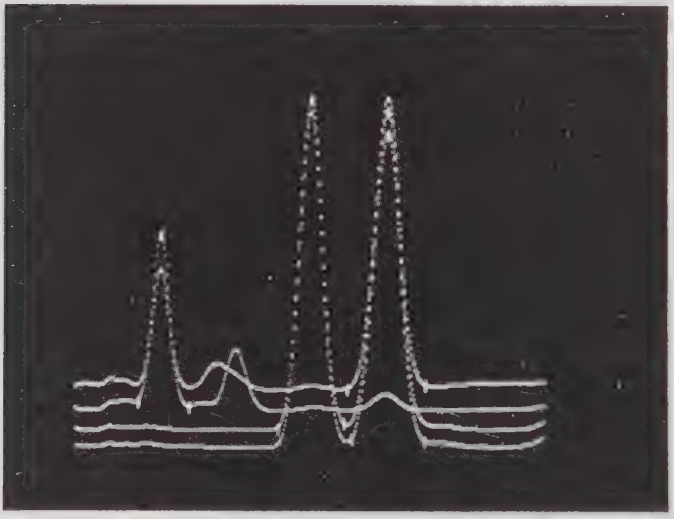

Fig. 3. Mass fragmentogram, displayed on the oscilloscope, obtained from derivatives of material extracted from rat urine. The major peaks are PCP, TCP and TCH. The mass spectrometer was set to detect *m/e* 338, 340 and 392 (two channels).

after a user-specified delay and continues until the data buffer is full or the user terminates the data collection. Determination of peak areas, peak heights and retention times occurs immediately after scanning is completed, following a spike rejection algorithm and curve smoothing[11]. The collected data are scanned according to the user-specified retention times. The peak areas or peak heights and retention times are displayed for each channel with interchannel peak area or height ratios. The data calculation can also be printed on the teletype. Background subtraction and baseline adjustments can be carried out on the collected data after collection. The system provides facilities for filing data under a sample number manually or automatically by sequential file numbers. Once the data have been stored on tape, the operator can recall any set of data for revaluation, etc. A library of user programs permits off-line plotting of groups of data automatically, or tabulations of comparisons between data sets. A sample of a user program for plotting the entire contents of the data tape is shown below.

```
100 PRINT "FIRST SAMPLE NUMBER",
110 INPUT Z
120 PRINT "LAST SAMPLE NUMBER",
130 INPUT U
140 FOR K = Z TO U STEP 1
150 REMARK READ IN THE DATA
160 TREAD K
170 REMARK PRINT THE CALCULATIONS FIRST
180 GOSUB 500
190 REMARK NOW PLOT THE DATA
200 PLOT -1
210 NEXT K
240 END
```

APPLICATION

Standard solutions

Five standard solutions were prepared by adding 100 μg/ml of tetrachloropyrocatechol (TCP) and 20–100 μg/ml each of pentachlorophenol (PCP) and tetrachlorohydroquinone (TCH) to urine collected from unexposed rats. A 1-ml volume of urine was then boiled for 2 h with 1 ml of concentrated hydrochloric acid and extracted four times with 1 ml of diethyl ether. The combined organic phase was evaporated to dryness and the residue dissolved in 0.3 ml of redistilled diethyl ether and 0.2 ml of N,O-bis(trimethylsilyl)acetamide (BSA). A 0.1–1.0-μl volume of the solution was injected into the GC–MS. Pentachlorophenol was given i.p. to 200–250-g male rats (Sprague–Dawley) in single doses of 10 mg/kg. The urine (pH not controlled) was collected at 0° in continuous nitrogen-flushed vessels. A 100-μg amount of tetrachloropyrocatechol was added to 1 ml of urine and the urine was subjected to the same extraction procedure as described above.

Analytical procedure

The components were separated on a silanized glass column packed with 5% SE-52 on Chromosorb W (60–80 mesh). The temperature of the oven and injection port were maintained at 200° and 240°, respectively. The flow-rate of the carrier gas (helium) was 20 ml/min. The ionizing potential was 50 eV and the trap current 60 μA, and the temperature of the ion source was 270°.

The mass numbers *m/e* 338, 340 and 392 were chosen for the analysis. Pentachlorophenol will respond at *m/e* 338 and 340, tetrachlorohydroquinone at *m/e* 340 and 392 and tetrachloropyrocatechol only at *m/e* 392. Two channels were focused on *m/e* 392 in order to calculate the response to tetrachlorohydroquinone at both *m/e* 340 and 392. These parameters were introduced into the program by answering the questions displayed on the oscilloscope.

The ratio of the peak heights or peak areas of pentachlorophenol (*m/e* 338) or tetrachlorohydroquinone (*m/e* 340, 392) to the internal standard tetrachloropyrocatechol (*m/e* 392) was calculated and plotted against the concentrations of pentachlorophenol and tetrachlorohydroquinone, respectively. New standard curves were

prepared each day. The amount of pentachlorophenol and tetrachlorohydroquinone in urine sample was calculated by extrapolation from the standard curves.

RESULTS

Internal standard

An internal standard is supposed to compensate for losses during extraction, differences in injection volume and evaporation during storage. Under the conditions used, tetrachloropyrocatechol was found to be suitable as its retention time did not coincide with those of other pesticides and/or impurities. A plotted recording from the analysis of a urine extract is shown in Fig. 4.

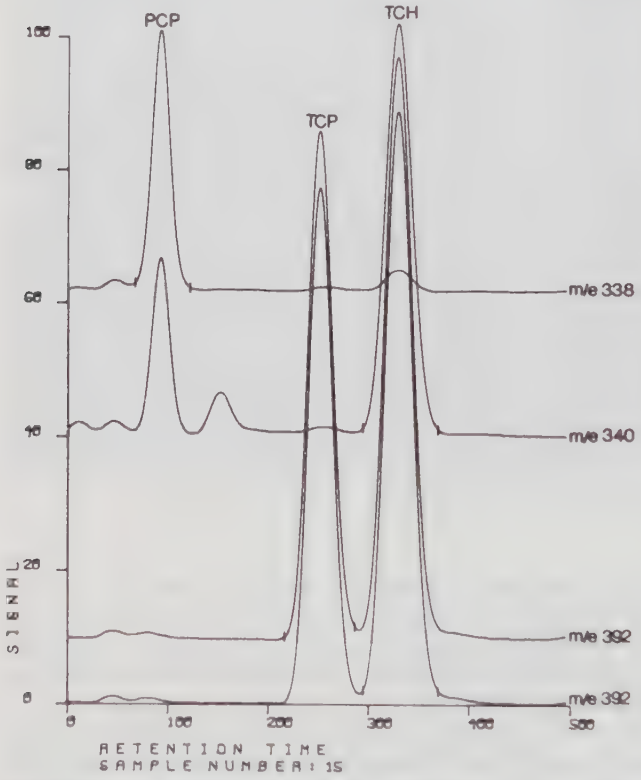

Fig. 4. Mass fragmentogram plotted on the plotter. Same peaks and mass numbers as in Fig. 3.

Standard curves

Standard curves for pentachlorophenol (*m/e* 338) and tetrachlorohydroquinone (*m/e* 340, 392) are shown in Figs. 5 and 6, obtained from calculations based on the peak height ratio and the peak area ratio, respectively. The slopes of the curves were found to be approximately the same from day to day.

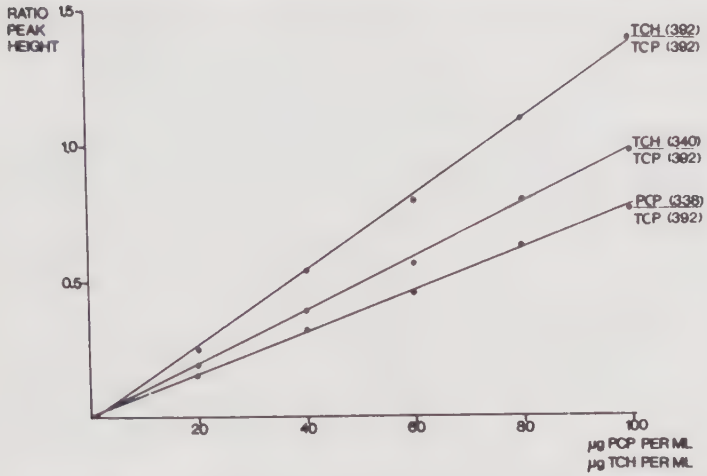

Fig. 5. Standard curve for the quantitative determination of PCP and TCH in rat urine by using the peak height ratios.

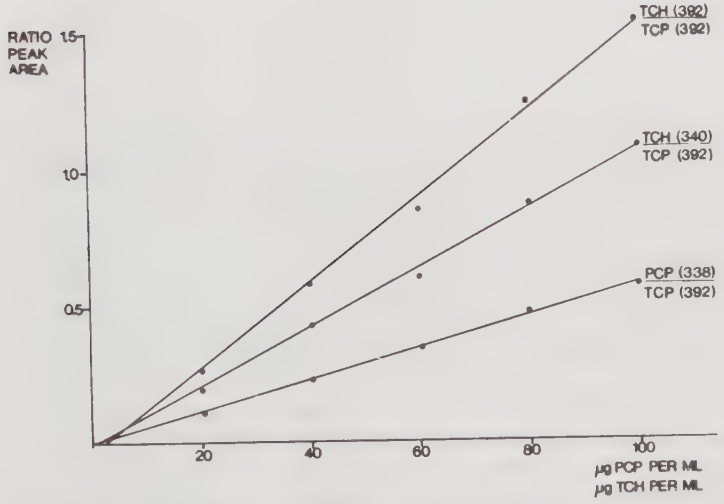

Fig. 6. Standard curve for the quantitative determination for PCP and TCH in rat urine by using the peak area ratios.

Urine excretion

The cumulative excretion of pentachlorophenol and its metabolite tetrachlorohydroquinone over a period of 72 h is shown in Fig. 7. The results are means for four rats and are in good agreement with those of total excreted radioactivity after administration of ^{14}C-labelled pentachlorophenol[12].

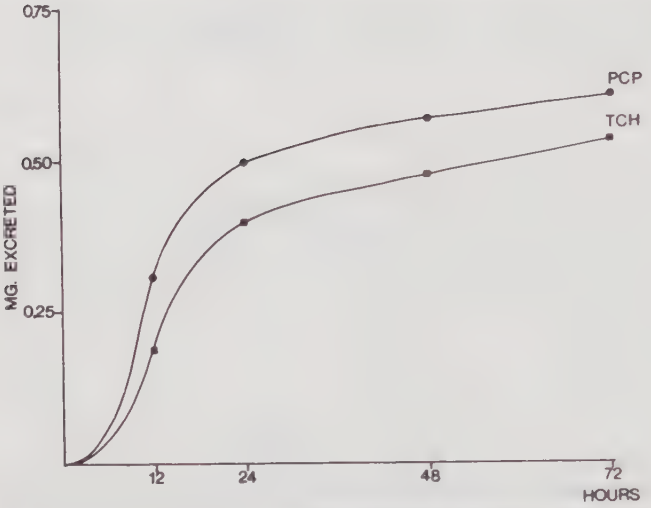

Fig. 7. Cumulative excretion of PCP and TCH in urine during 72 h after i.p. administration of PCP (10 mg/kg). Average from four rats.

DISCUSSION

Although previous techniques for multiple ion detection (MID) have been used with advantage in qualitative and quantitative mass fragmentography, the approach described in this paper offers many advantages. The MID described by Hammar and Hessling[2] has a mass range of 20 % and that described by Hammar[6] a mass range of 30% of the lowest mass within which different masses can be focused. The accelerating voltages required to bring the selected masses into focus were added to the basic accelerating voltage. The curves for individual masses were made continuous by the utilization of a sample-hold circuit for each mass channel and by using one galvanometer per channel and individual channel controls for the amplification of the ion signals.

The MID system described in this paper has a mass range of 30 % calculated on the lowest mass. However, in contrast to previous systems, the accelerating voltage is digitally controlled by the computer over a D/A converter. One of the main advantages is the digital signal treatment, which offers a more satisfactory control of baseline and gain and the possibility of computation of retention times and peak ratios. The system is not limited to hardware-implemented channels and therefore permits expansion of the number of channels without hardware modification. The number of channels is dependent only on mass storage limitation and the data collection rate. The system permits observations of the peaks on the oscilloscope as they emerge from the gas chromatograph.

Furthermore, the data and the calculations are presented as hard copy on the teletype. All data as well as the calculated peak values and ratios are stored on inexpensive magnetic tape for future use. The system permits calculations based on

both peak areas and peak heights; the latter seems to be more satisfactory for quantitation in biological samples.

Mass fragmentography has in recent years emerged as an important technique for the study of both drugs and biologically occurring compounds. It offers special advantages in the study of metabolism and is a specific means of detection and quantitation in the picogram to nanogram range[13]. It is hoped that the system described in this paper will facilitate considerably the use of mass fragmentography.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council (14X-4041, 40X-199, 40Y-2375 and 70E-3743), the Swedish Environment Protection Board (7-1/72), the Bank of Sweden Tercentenary Fund (68/53), the Wallenberg Foundation, the National Institute of Mental Health (MH 12007) and by funds from the Karolinska Institutet.

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Arch. Toxicol. 32, 271—281 (1974)
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Metabolism of Pentachlorophenol

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Received May 17, 1974

Abstract. Excretion of pentachlorophenol-^{14}C in the urine of rats and mice after oral and intraperitoneal administration (10 to 25 mg/kg) was studied. 41 to 43 % of the excretion activity was found to be present as unchanged pentachlorophenol. By means of gas chromatography-mass spectrometry, one metabolite was identified as tetrachlorohydroquinone, representing 5 and 24 % of the excreted activity in rats and mice, respectively. Conjugation with glucuronic acid could not be demonstrated by enzymatic hydrolysis since tetrachlorohydroquinone was found to be a potent inhibitor of β-glucuronidase, the I_{50} being 1.6 to 2.0 $\times$ 10^{-6} M. Boiling the urine with hydrochloric acid was shown to convert all of the excreted activity to pentachlorophenol and tetrachlorohydroquinone, the latter representing 43 and 46 % in rats and mice, respectively. Tetrachlorohydroquinone was found in the urine of workers occupationally exposed to pentachlorophenol.

Key words: Pentachlorophenol — Excretion — Metabolism — Tetrachlorohydroquinone — β-glucuronidase.

Zusammenfassung. Die Ausscheidung von ^{14}C-Pentachlorphenol im Harn von Ratten und Mäusen wurde nach oraler und intraperitonealer Verabreichung in Dosen von 10 bis 25 mg/kg untersucht. 41 bis 43 % der ausgeschiedenen Aktivität waren unverändertes Pentachlorphenol. Mittels kombinierter Gaschromatographie und Massenspektroskopie konnte ein Metabolit, der 5 bzw. 24 % der ausgeschiedenen Aktivität bei Ratten bzw. Mäusen ausmacht, als Tetrachlorhydrochinon identifiziert werden. Das Vorhandensein von Glucuronsäure-Konjugaten ließ sich nicht durch enzymatische Hydrolyse nachweisen, da Tetrachlorhydrochinon ein starker β-Glucuronidase-Hemmer (I_{50} = 1,6 bis 2,0 $\times$ 10^{-6} M) ist. Kochen des Harns mit HCl wandelte die gesamte ausgeschiedene Aktivität in Pentachlorphenol und Tetrachlorhydrochinon um. Davon entfielen 43 (Ratten) bzw. 46 % (Mäuse) auf Tetrachlorhydrochinon. Tetrachlorhydrochinon konnte auch im Harn von Arbeitern nachgewiesen werden, die beruflich mit Pentachlorphenol in Kontakt kommen.

Schlüsselwörter: Pentachlorphenol — Ausscheidung — Metabolismus — Tetrachlorhydrochinon — β-Glucuronidase.

Pentachlorophenol (PCP) and its sodium salt are widely used as molluscides, wood preservatives, fungicides, pesticides, and herbicides.

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Available toxicological and analytical data have been reviewed by Bevenue and Beckman (1967).

Human exposure occurs frequently and there are several reports of intoxications in the literature; e.g. an epidemic with two deaths that occurred in a nursery in St. Louis after the use of pentachlorophenol as a mildew preventive in a laundry detergent (Barthel *et al.*, 1969).

Despite the importance of the problem, little work has been devoted to the study of the detailed metabolism of PCP. From experiments in the rabbit and the rat it has been proposed that PCP is excreted unchanged and unconjugated from the body (Deichmann *et al.*, 1942; Betts *et al.*, 1955). In the mouse, however, PCP has been found to be excreted both free and conjugated, unconjugated tetrachlorohydroquinone has been identified as one excretion product in this species (Jacobsson and Yllner, 1971).

In preliminary experiments, we noticed that when PCP-^{14}C was administered intraperitoneally to rats, only about 40% of the radioactivity excreted in the urine could be recovered after adjustment of the urine to pH 2 and extraction with ether. Several other extraction methods (Bevenue *et al.*, 1966; Rudling, 1970; Rivers, 1972), when tested, gave approximately the same figure.

By contrast, when PCP was added to urine from nonexposed rats and extracted recovery was almost complete.

This discrepancy suggested that at least part of the urinary activity consists of other compounds and prompted us to reinvestigate the metabolic fate of pentachlorophenol.

Experimental

Reference Compounds

Pentachlorophenol-^{14}C(U) was obtained from AB Atomenergi, Studsvik. The specific activity was 1.14 mc/mM or 4.32 μc/mg and by radiochromatogram scanning, after paper chromatography with *n*-butanol-glacial acetic acid-water (4:1:5) as solvent, it was shown to be 100% isotopically pure. For the experimental work, labeled pentachlorophenol was diluted to suitable activity with unlabeled pentachlorophenol. Other reference compounds and chemicals of reagent grade were obtained commercially. Unlabeled pentachlorophenol, upon analysis with gas chromatography-mass spectrometry, proved to contain small amounts of tetra- and trichlorophenols.

Abbrevations Used

PCP: pentachlorophenol
TCH: tetrachlorohydroquinone
TCP: tetrachloropyrocatechol

Animal Experiment and Excretion of Labeled Materials

Mice (NMRI, 20 to 30 g) and rats (Sprague-Dawley, 200 to 300 g) were given PCP-^{14}C (10 to 25 mg/kg), dissolved in olive oil or in propylene glycol, either intraperitoneally or *per os*. After administration, the animals were placed in glass metabolism cages designed for the separate collection of urine and feces. The urine was collected in a vessel continuously flushed with nitrogen. A slow current of dry, CO_2-free air was constantly passed through the cages and conducted from them through two absorption vessels containing a 15% solution of ethanolamine in methanol in order to absorb CO_2. The animals were allowed water and food *ad libitum* during the course of the experiment. Radioactivity measurements were performed with a liquid scintillation spectrometer (Packard Tri-Carb, Mod. 3375) using "Aquasol NEN" as the liquid scintillator.

Paper Chromatography, Thin-Layer Chromatography and Radiochromatogram Scanning

Chromatographic separation of metabolites of PCP-^{14}C in urine and comparisons with references were carried out on Whatman 3 MM paper with four different solvents (I to IV) and on precoated silicagel F 254 Merck plastic sheets with one solvent (V):

I	Methanol-water-concentrated ammonia	(8:1:1)
II	Acetone-water-concentrated ammonia	(8:1:1)
III	Isoamyl alcohol-water-concentrated ammonia	(6:1:3)
IV	*n*-Butanol-water-glacial acetic acid	(4:5:1)
V	Methyl acetate-isopropanol-concentrated ammonia	(9:7:4)

Thin-layer plates were washed by successively allowing freshly distilled methanol and methyl acetate to ascend to the upper edges. Before use, the plates were activated 30 min at 110 °C.

The urine was applied on paper and plate either directly or after the following different hydrolytic treatments:

a) *Treatment with β-glucuronidase:* 2 ml of urine was incubated overnight at 37 °C with bacterial β-glucuronidase (Sigma type I) in 0.075 M phosphate buffer at pH 6.8; the enzyme activity was increased by addition of one drop of chloroform.

b) *Treatment with Sulfatase:* 2 ml of urine was incubated overnight at 37 °C with arylsulfatase (Sigma type III) in 0.2 M sodium acetate buffer at pH 5.0.

c) *Treatment with Hydrochloric Acid:* 1 ml of urine was mixed with 1 ml concentrated hydrochloric acid in an ampoule which was then sealed and either left overnight at room temperature or placed in an oven at 100 °C for 1 h. The mixture was then neutralized with 10 N sodium hydroxide.

Radioactive areas, on TLC or paper, were located by using a Packard radiochromatogram scanner mod. 7201.

Radio-Gas Chromatography and Gas Chromatography-Mass Spectrometry

Radio-gas chromatography was performed on an F&M model 400 apparatus equipped with a flame ionization detector and connected to a Packard model 893 gas chromatography proportional counter. The column was a 1.6 m × 3 mm (i.d.) glass U-tube packed with 3% SE-30 on Gas Chrom P, 100/120 mesh (Applied Science Lab. Inc.). Analyses were made at a column temperature of 125° with an argon carrier gas flow of 60 ml/min. The temperature of the vaporizer and flame detector was 200°.

The combustion chamber was kept at 800°. The flow of the propane quench gas was 3 ml/min.

Gas chromatography-mass spectrometry was performed on an LKB model 9000 gas chromatograph-mass spectrometer (LKB-Produkter, Bromma, Sweden). The separations were made on a 1.6 m × 3 mm (i.d.) silanized glass column, packed with 3 % XE-60 on Gas Chrom Q (Applied Science) maintained at a temperature of 160°. The flow rate of helium carrier gas was 25 ml/min. The ionizing potential and trap current were 70 eV and 60 μA, respectively. The temperature of the flash heater was 210° and the ion source was kept at 250°.

Methods Used for Identification of Metabolites

Analytical Procedure I: The urine was lyophilized and the residue redissolved in a few ml of methanol and applied as a band on a paper and developed by the descending method in solvent system IV. The relevant areas of the paper were cut separately into small pieces and eluted by shaking 4 h with freshly redistilled methanol containing 2 % concentrated ammonia. The two solutions were centrifuged, dried with anhydrous sodium sulphate, filtered and evaporated under nitrogen at 40 °C, to about 1 ml. The residual fractions were transferred to a silicagel plate which, after developing in solvent system V, were subjected to radio scanning.

Areas showing activity were removed from the plate by scraping and eluted by shaking overnight with methanol containing 2 % concentrated ammonia. The suspension was centrifugated 15 min at 3000 r/min, dried by shaking 4 h with anhydrous sodium sulfate, and evaporated under nitrogen at 40 °C to a volume of 0.3 ml. The solution was divided into three 0.1 ml fractions which were evaporated under nitrogen. The residues were derivatized and submitted to gas chromatography-mass spectrometry.

Analytical Procedure II: A urine sample (20 ml) was divided into two portions. The first 10 ml (II A) were adjusted to pH 2 with 2 N hydrochloric acid and extracted with three 20 ml aliquots of ether. The ether extracts were combined, dried with anhydrous sodium sulfate, filtered and evaporated under nitrogen. The residue was dissolved in methanol; a fraction of this solution was applied on thin-layer and developed. The other part was methylated and assayed by gas chromatography-mass spectrometry. The remaining aqueous phase was made alkaline to pH 10 with concentrated ammonia in order to avoid volatilization of the acidic phenols and concentrated under vacuum. The residue was submitted to thin-layer chromatography.

The second 10 ml (II B) of the urine sample were treated with 5 N hydrochloric acid at 100 °C for 1 h in a sealed ampoule; after cooling, the mixture was adjusted to pH 2, extracted and handled as described above. The aqueous phase was not further investigated.

Derivatization

Methyl ethers were prepared by dissolving the residue in 0.2 ml of methanol and treating the solution with diazomethane in ether during 10 h at room temperature. The solvent was evaporated under nitrogen and the residue redissolved in methanol. *Trimethylsilyl ethers* were prepared by adding successively 50 μl of pyridine (distilled from and stored over sodium hydroxide), 15 μl of trimethylchlorosilane, and 50 μl of hexamethyldisilazane to the residue. The reaction mixture was left overnight in a stoppered tube and the solvent evaporated under vacuum. The residue was dissolved in redistilled ethyl acetate. *Acetyl ethers* were prepared by adding 1 ml pyridine and 0.5 ml acetic anhydride to the residue. The reaction mixture was left overnight in a

stoppered tube, the solvent was evaporated at 40 °C under nitrogen, and the residue dissolved in ethyl acetate.

Enzyme Inhibition Studies

β-glucuronidase activity was studied using phenolphtalein-β-glucuronide or p-nitrophenyl-β-D-glucuronide as substrate, according to Sigma Chem. Comp. 1973(a). Before incubation varying amounts of PCP or TCH were added and after incubation the optical density was read in a Beckman DB spectrophotometer at 540 and 435 mμ for the two substrates, respectively.

Aryl sulphatase (Sigma type III) activity was studied, using nitrocatechol sulfate as substrate according to Sigma Chem. Comp. 1973(b). Optical density was read at 515 mμ.

Analysis of Urine from Exposed Humans

24 h urine specimens were collected in the morning the day after exposure from two occupationally exposed spraymen.

Suitable amounts of TCP, as an internal standard, were added to a 50 ml volume of urine. The urine was boiled for 30 min with 5 ml of concentrated hydrochloric acid. After cooling, 8 ml of 10 M NaOH was added and the mixture was extracted with an aliquot of diethylether. The organic phase was discarded. 3 ml of concentrated hydrochloric acid was added and the mixture was extracted with an aliquot of diethylether. The organic phase was carefully evaporated until dry; methylated with diazomethane, evaporated until dry and redissolved in 0.5 ml hexane. The solution was then cleaned by washing with 20% water in methanol, as described by Cranmer and Freal 1970.

The analysis was performed by means of mass fragmentography on an LKB model 9000 gas chromatograph-mass spectrometer equipped with computer control and digital signal processing (Elkin *et al.*, 1973). The separation was made on a 2.2 m × 3 mm (i.d.) silanized glass column packed with 10% Carbowax 20 M terephtalic acid on Varaport 30 (100 to 120 mesh) maintained at a temperature of 180 °C. The flow rate of helium carrier gas was 25 ml/min. The ionizing potential and trap current were 50 eV and 60 μA, respectively.

The mass numbers m/e 237, 259, 261, and 265 were chosen for the analysis. PCP will respond at all mass numbers, TCH and TCP only at m/e 259 and 261.

Results

The urinary excretion of total radioactivity is presented in Fig. 1. No radioactivity was recovered in the expired air.

40 h after *intraperitoneal* injection in rats and mice, most of the radioactivity was recovered in the urine. When given *orally*, the radioactivity recovered from urine was about 50% lower in mice than in rats.

Paper and thin-layer chromatographic data from untreated rat urine for the different solvent systems are found in Table 1.

The radioactivity is distributed in 3 peaks after both thin-layer and paper chromatography with solvent systems I and II. On paper chromatography with system I or II, the second peak is consistent with TCH, and the last one with PCP. On thin-layer chromatography the first peak is consistent with TCH, the second with TCP, and the third with PCP.

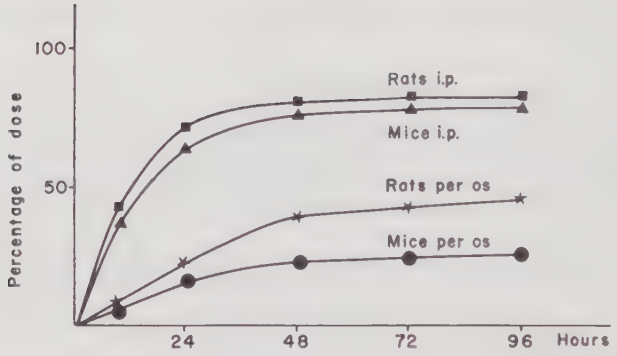

Fig. 1. Cumulative excretion of radioactivity in the urine after oral and i.p. administration of a single dose of pentachlorophenol-^{14}C (25 mg/kg) to mice and rats

Table 1. Distribution of the radioactivity after paper or thin-layer chromatography in rat and mouse urine

	Paper chromatography Solvent system				TLC
Compound	I	II	III	IV	V
PCP	0.78	0.96	0.81	0.80	0.82
TCH	0.64	0.67	0.00	0.62	0.00
TCP	0.76	0.85	0.75	0.78	0.50
	0.02	0.03	0.02	0.62	0.02
Urine	0.61	0.65	0.80	0.80	0.50
	0.79	0.95			0.82

With solvent systems III and IV, only two peaks appear, the Rf-values corresponding to TCH and PCP and/or TCP, respectively. Since the distribution pattern on thin-layer and paper chromatograms were similar, regardless of route of administration, thin-layer chromatography and intraperitoneal injections were chosen for the continued experimentation.

Table 2 shows the distribution of radioactivity between the three peaks when rat and mouse urine was applied either untreated or after the different hydrolytic treatments. The percentage of the activity corresponding to the peak at Rf 0.02 was higher in mice than in rats. In both species the activity distribution remained unchanged after treatment of the urine with β-glucuronidase and sulfatase or hydrochloric acid at room temperature.

Table 2. Thin-layer chromatography distribution of the radioactivity before and after treatment of the urines of animals injected i.p. with PCP-^{14}C

Animal	Treatment of the urine	Percentage of radioactivity corresponding to peaks at Rfs 0.02	0.50	0.82
Rat	—	5	52	43
	β-glucuronidase	4	53	43
	Sulfatase	7	50	43
	HCl room temp.	8	46	46
	HCl 100 °C	43	—	57
Mouse	—	24	35	41
	β-glucuronidase	20	39	41
	Sulfatase	25	33	42
	HCl room temp.	22	35	43
	HCl 100 °C	46	—	54

Table 3. Retention times, on 3 % SE 30 column, at 125 °C of the methyl-, acetyl-, and trimethylsilyl derivatives of fractions A and B and reference compounds

	Methyl derivatives	Acetyl derivatives	Trimethylsilyl derivatives
PCP	3′25″	8′10″	9′20″
TCH	3′25″	14′	15′30″
Fraction A	—	14′	15′30″
Fraction B	3′20″	8′10″	9′20″

However, the peak at Rf 0.50 completely disappears after treatment with concentrated hydrochloric acid at 100 °C. In the same process the activities at Rfs 0.02 and 0.82 increase, more pronounced for the peak at Rf 0.02. The distribution of the activity between peaks at Rfs 0.02 and 0.82 thus becomes nearly identical in rats and mice.

Analytical Procedure I: Paper chromatography of the urine upon scanning showed the distribution of radioactivity in two peaks at Rfs 0.62 (A) and 0.80 (B), respectively. When the eluates of the corresponding two spots were rerun on thin-layer, fraction A gave a single peak at Rf 0.02 and fraction B a peak at Rf 0.80. Acetylation or trimethylsilylation of fractions A and B, upon gas chromatography, revealed peaks with a retention time consistent with TCH for fraction A and with PCP for fraction B (Table 3). The mass spectra of the reference compounds and of the effluents from the gas chromatography column confirm the identification.

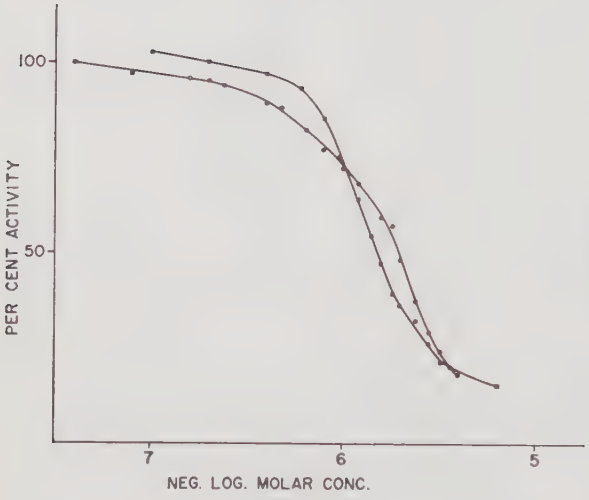

Fig. 2

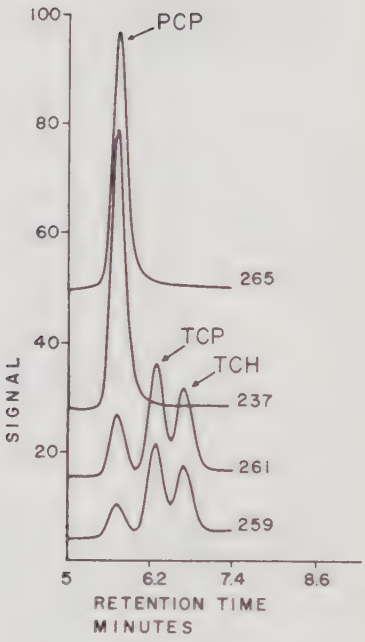

Fig. 3

Fig. 2. β-glucuronidase activity as a function of tetrachlorohydroquinone concentration for two different substrates in phosphate buffer, pH 6.8

Fig. 3. Mass fragmentogram obtained from derivatives of material extracted from urine from workers occupationally exposed to pentachlorophenol. The mass spectrometer was set to detect m/e 237, 259, 261, and 265

Analytical Procedure II: In the ether extraction of acidified urine (portion II A) 42% of the radioactivity present in the urine was recovered. Scanning of this extract after TLC reveals one major peak at Rf 0.82 and a very small one at Rf 0.02 (less than 1%). Examination of the remaining aqueous phase gives a major peak at Rf 0.50 and another one at Rf 0.02. In the extract of urine boiled with HCl (portion II B), 92% of the total radioactivity was recovered.

Scanning of this extract after TLC revealed two peaks of almost equal height at Rfs 0.02 and 0.82. Gas chromatography-mass spectrometry of the ether extracts from both unhydrolyzed (II A) and hydrolyzed (II B) urines proved the presence of two main compounds, PCP and TCH, TCH being present in a much larger quantity in the hydrolyzed urine extract. In addition, with both extraction procedures after methylation, the gas chromatographic effluent contained small amounts of two other compo-

nents. One had a molecular ion (M^+) of 244 and contained four chlorine atoms; the second had a molecular ion (M^+) of 240 and contained 3 chlorine atoms.

Enzyme Inhibition Studies: Concentrations of PCP up to 100 µg/ml (3.75×10^{-4} M) in the β-glucuronidase incubations systems gave no inhibition.

Concentrations of PCP or TCH up to 250 µg/ml (9.4×10^{-4} and 1.0×10^{-3} M, respectively) in the aryl sulfatase system gave no inhibition.

The inhibitory effect of different concentrations of TCH in the two β-glucuronidase systems are shown in Fig. 2, the I_{50} being in the range 1.6 to 2.0×10^{-6} with phenolphtaleinglucuronidate or p-nitrophenol-β-D-glucuronide as substrate.

Analysis of Urine from Exposed Humans

Upon mass fragmentography of the urine extract, peaks corresponding to both PCP, TCH, and the internal standard TCP were found (Fig. 3).

Discussion

Intraperitoneal injection of PCP-^{14}C in mice and rats was followed by a rapid excretion of the major part of the radioactivity in urine, about 70% being recovered after 24 h. Urinary excretion was lower after oral administration, as has also been noted in the rat by Larsen *et al.*, 1972. In the present experiment, excretion was found to be higher in the rat than in the mouse.

Earlier studies (Deichmann *et al.*, 1942; Betts *et al.*, 1955) found PCP to be excreted mainly unchanged and unconjugated. The present study demonstrated that only around 40% of the excreted activity in both mice and rats is present as unconjugated PCP. Jacobsson and Yllner (1971) reported the occurrence of conjugated PCP. The nature of the conjugate was not investigated. In addition, unconjugated TCH was found to represent about 40% of the urinary excretion in mice. TCH was identified by means of isotope dilution analysis.

In our investigation, paper and thin-layer chromatography showed the presence of two other major components beside PCP. By means of gas chromatography-mass spectrometry, unconjugated TCH could be demonstrated to be one of those representing 24% of the 24 h of activity in the mouse, but only 5% in the rat.

A second major metabolite was demonstrated with paper or thin-layer chromatography of untreated urine or in the aqueous phase after ether extraction of the urine. The chromatographic behavior of this component was very similar to that of tetrachloropyrocatechol (Table 1), but upon boiling of the urine with hydrochloric acid, the component in

question decomposed into TCH and PCP, both of which could be identified by gas chromatography-mass spectrometry. In all likelihood, therefore, this component is a mixture of the conjugates of both PCP and TCH.

Attempts to hydrolyze the conjugates enzymatically were unsuccessful. When the activity of both sulfatase and β-glucuronidase was tested, sulfatase was found to be uninfluenced by both PCP and TCH, whereas the β-glucuronidase was found to be inhibited by TCH, but not by PCP. Under the experimental conditions, the I_{50} for TCH was found to be 1.6 to 2.0×10^{-6} M (0.4 to 0.5 μg/ml).

This inhibitory activity seemed to be remarkably high as compared to other known inhibitors of β-glucuronidase (Mills *et al.*, 1952; Dutton, 1966). The amount of TCH in the urine from rats given PCP, as described, is in the range 10 to 100 μg/ml (Elkin *et al.*, 1973) and thus high enough to explain the lack of β-glucuronidase activity under the experimental circumstances.

Two minor components were found and identified from mass spectral data. Tetrachlorophenol, which was proven to occur as a contaminant in the analytical grade PCP used in the experiments, and trichlorohydroquinone, which in all probability is a metabolite of the latter.

The fact that PCP is metabolized and that both parent compound and metabolite partly occur as conjugates should be taken into account when urinary excretion is used as a measure of occupational exposure to PCP. Boiling with concentrated HCl accomplishes a complete hydrolysis of the conjugates for the quantification of PCP and TCH. The use of mass fragmentographic techniques (Elkin *et al.*, 1973) seems to offer the best analytical possibilities for the study of occupational exposure.

Acknowledgements. This work was supported by grants from the Swedish Environment Protection Board (7-1/72), the Bank of Sweden Tercentenary Fund (68/53) the Wallenberg foundation and the National Institute of Mental Health (MH 12007).

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Arch. Toxicol. 37, 81–87 (1977)

Archives of
TOXICOLOGY
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Original Investigations

Inhibition of β-Glucuronidase by Chlorinated Hydroquinones and Benzoquinones

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Abstract. An earlier study of the metabolism of pentachlorophenol has shown that a metabolite, tetrachloro-p-hydroquinone, possessed pronounced inhibitory action on the activity of β-glucuronidase from bacterial origin. Several other chlorinated hydroquinones and benzoquinones have now been studied with regard to their ability to inhibit β-glucuronidase of various origin in vitro and in vivo.

All the studied chlorinated hydroquinones and benzoquinones were found to be potent inhibitors of β-glucuronidase of bacterial origin. D-glucaric acid-1.4-lactone was included for comparison and was found to be less active than the other studied compounds. The inhibition was found to be competitive in nature.

No inhibitory effect of the benzo- and hydroquinones studied in vitro or in vivo could be demonstrated on β-glucuronidase from livers. The result calls for precaution when using bacterial β-glucuronidase to split urinary conjugates of glucuronic acid.

Key words: β-Glucuronidase – Inhibition – Chlorobenzoquinones – Chlorohydroquinones.

Zusammenfassung. Eine frühere Studie des Metabolismus von Pentachlorphenol hat gezeigt, daß ein Metabolit, Tetrachlor-p-hydrochinon, eine ausgesprochene Hemmwirkung auf die Aktivität der Bakterien-β-Glucuronidase besitzt. Verschiedene andere chlorierte Hydrochinone und Benzochinone sind jetzt auf ihre Fähigkeit, β-Glucuronidase anderen Ursprungs zu hemmen, in vitro und in vivo, untersucht worden.

Alle die untersuchten chlorierten Hydrochinone und Benzochinone zeigten sich als starke Hemmer der Bakterien β-Glucuronidase. D-Glucarsäure-1.4-Lakton wurde zum Vergleich einbezogen und wurde weniger aktiv als die anderen Verbindungen befunden. Die Hemmung war kompetitiver Art.

Kein Hemmungseffekt von Hydro- oder Benzochinonen konnte in vitro oder in vivo auf Leber-β-Glucuronidase gezeigt werden. Das Resultat mahnt zur Vor-

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sicht, wenn Bakterien β-Glucuronidase zur Spaltung von Harnkonjugaten der Glucuronsäure angewandt wird.

During a study of the metabolism of pentachlorophenol in rats it was found that part of the urinary excretion of both pentachlorophenol and its metabolite tetrachloro-p-hydroquinone was in the form of conjugates. Usual breaking of the suspected glucuronide conjugates by treatment with β-glucuronidase of bacterial origin was, however, unsuccessful and it could be shown that this was due to a potent inhibitory action by tetrachloro-p-hydroquinone on the enzyme activity (Ahlborg et al., 1974).

Further studies of the metabolism of other chlorinated phenols have indicated the occurrence of several chlorinated hydroquinones as urinary metabolites (Ahlborg and Larsson, 1977). Chlorinated benzoquinones, such as chloranil (tetrachloro-p-benzoquinone), have been shown to occur as biodegradation products of pentachlorophenol i.e. in water (Kuwahara et al., 1965, 1966a, b) and as a urinary metabolite in rabbits exposed to pentachlorophenol (Tashiro et al., 1970).

The occurrence of β-glucuronidase in different tissues including blood is well documented (Hayashi, 1964). The importance of its occurrence and distribution is, however, not well understood and the effects of a possible inhibition in vivo is not easily predicted.

This study treats the possible inhibitory action of several chlorinated hydroquinones and benzoquinones, in vitro and in vivo.

Materials and Methods

Reference Compounds.

Tetrachloro-p-hydroquinone,	mw 247.89,	Eastman Kodak Co., USA
Tetrachloro-p-benzoquinone,	mw 245.88,	J. T. Baker Chem., Holland
Tetrachloropyrocatechol,	mw 247.91,	Schuchardt, Germany
2.3.5.6-Tetrachlorophenol,	mw 231.89,	Fluka AG., Switzerland
Trichloro-p-hydroquinone,	mw 213.46,	was synthesized as described by Ahlborg and Larsson, 1977
Trichloro-p-benzoquinone,	mw 211.44,	was prepared by oxidation of trichloro-p-hydroquinone with $Na_2Cr_2O_7$ as described by Graebe (1868). Purity and identity was checked with gas chromatography–mass spectrometry
2.5-Dichloro-p-benzoquinone,	mw 176.99,	Eastman Kodak Co., USA
2.6-Dichloro-p-benzoquinone	mw 176.99,	Eastman Kodak Co., USA
2.5-Dichloro-p-hydroquinone,	mw 179.01,	was prepared by reduction of the corresponding benzoquinone
2.6-Dichloro-p-hydroquinone,	mw 179.01,	was prepared by reduction of the corresponding benzoquinone
2.3.5-Trichlorophenol,	mw 197.45,	Fluka AG., Switzerland
2.3.6-Trichlorophenol,	mw 197.45,	Fluka AG., Switzerland
3.6-Dichloro-2.5-dihydroxy-1.4-benzoquinone (chloranilic acid),	mw 208.99,	Fluka AG., Switzerland
2-Chloro-p-hydroquinone,	mw 144.56,	Fluka AG., Switzerland

Hydroquinone,	mw 110.11,	Fluka AG., Switzerland
D-glucaric acid-1.4-lactone,	mw 192,10,	Sigma Chem. Co., USA
Phenolphtalein-β-glucuronide,	mw 516.40,	(anhydrous), Sigma Chem. Co., USA
p-Nitrophenyl-β-D-glucuronide,	mw 315.20,	(anhydrous), Sigma Chem. Co., USA

Inhibition of β-Glucuronidase of Bacterial Origin. β-Glucuronidase activity was studied using phenolphtalein-β-glucuronide or p-nitrophenyl-β-D-glucuronide as substrate, according to Sigma Chem. Co. (1973). The incubations were carried out at pH 6.8. Before incubation, varying amounts of the chlorinated compounds in ethanol were added and after incubation the optical density was read in a Beckman DB spectrophotometer at 540 and 435 nm for the two substrates, respectively. Controls were run with corresponding volumes of ethanol. The β-glucuronidase used was Sigma type 1 β-glucuronidase (62,000 units/g) supplied as a bacterial powder from E. coli culture fluid.

Inhibition of β-Glucuronidase from Liver. The effect of tetrachloro-p-hydroquinone and tetrachloro-p-benzoquinone was studied using bovine liver β-glucuronidase Sigma type B-1 (903,000 units/g) and the effect compared with that of D-glucaric acid-1.4-lactone. Phenolphtalein-β-glucuronide was used as substrate. The action was studied under different pH conditions using either acetate or phosphate buffers.

For comparison the effects were also studied on mouse liver homogenates prepared as described by Niwa et al. (1972) as well as on microsomal fractions from rat liver prepared in the following way. Male Spraque-Dawley rats (weight 300 g) were given phenobarbital sodium (75 mg/kg) for 3 consecutive days. They were starved over night and killed by decapitation. The livers were removed and weighed in 0.25 M sucrose and homogenized with a Potter-Elvehjem homogenizer. The homogenate was diluted with 0.25 M sucrose to 5 times the weight of the livers and centrifuged 2 min at 500 g and 8 min at 13,300 g. The pellet was discarded and the supernatant centrifuged at 105,000 g for 60 min. The pellet was then suspended in 0.15 M KCl and recentrifuged 30 min at 105,000 g. The new pellet was suspended in 0.25 M sucrose to a final concentration of 0.5 g liver/ml. All procedures were carried out at 0—4° C.

Microsomal protein was determined as described by Lowry et al. (1951) with the modifications recommended by Miller (1959). The incubations were carried out in acetate buffer at pH 4.5, and activity determinations were performed as described earlier.

In vivo Inhibition of β-Glucuronidase. In order to test a possible in vivo effect of tetrachloro-hydroquinone, the compound was administered to male NMR mice, weighing 20—25 g in a dose of 25 mg/kg intraperitoneally.

After 6 h the mice were sacrificed and liver homogenates prepared according to Niwa et al. (1972). The β-glucuronidase activity in the liver homogenate was determined as described earlier using p-nitrophenyl-β-D-glucuronide as substrate.

Results

Inhibition of β-Glucuronidase of Bacterial Origin

The activity of bacterial β-glucuronidase at different concentrations of some of the studied chlorinated hydroquinones and benzoquinones is shown in Figure 1. The activity is expressed in percentage of the enzyme activity with no inhibitor present.

The I_{50} for the compounds showing inhibitory action within the concentration used are given in Table 1.

For the other studied compounds no inhibition of β-glucuronidase could be shown using concentrations up to the level indicated in Table 2.

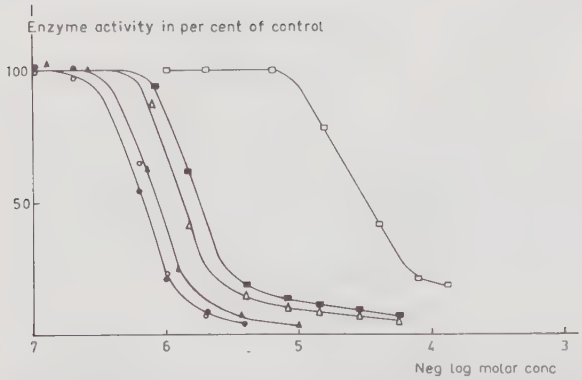

Fig. 1. The inhibitory effect for some of the studied compounds. Phenolphtalein-β-glucuronide was used as substrate and the enzyme source was bacterial β-glucuronidase, pH 6.8. ○—○ tetrachloro-p-hydroquinone; ●—● tetrachloro-p-benzoquinone; ▲—▲ trichloro-p-hydroquinone; △—△ 2.5-dichloro-p-hydroquinone; ■—■ 2.6-dichloro-p-hydroquinone; □—□ tetrachloropyrocatechol

Table 1. I_{50}-values of different chlorinated hydroquinones and benzoquinones with phenolphtalein-β-glucuronide as substrate and bacterial β-glucuronidase as enzyme source, pH 6.8

D-glucaric acid-1.4-lactone	7.9×10^{-4} M
Tetrachloro-p-hydroquinone	4.7×10^{-7} M
Tetrachloro-p-benzoquinone	6.3×10^{-7} M
Trichloro-p-hydroquinone	6.5×10^{-7} M
Trichloro-p-benzoquinone	6.6×10^{-7} M
2.5-Dichloro-p-hydroquinone	1.3×10^{-6} M
2.5-Dichloro-p-benzoquinone	6.6×10^{-7} M
2.6-Dichloro-p-hydroquinone	1.7×10^{-6} M
2.6-Dichloro-p-benzoquinone	7.2×10^{-7} M
Chloro-p-hydroquinone	1.1×10^{-5} M
p-Hydroquinone	8.9×10^{-7} M
1.4-benzoquinone	1.4×10^{-5} M
Tetrachloropyrocatechol	3.2×10^{-5} M

Table 2. The highest tested concentrations of different chlorinated compounds with no inhibitory effect on bacterial β-glucuronidase with phenolphtalein-β-glucuronide as substrate, pH 6.8

Compound	Molar concentration
Pentachlorophenol	3.75×10^{-4}
2.3.4.5-Tetrachlorophenol	0.96×10^{-4}
2.3.5.6-Tetrachlorophenol	0.96×10^{-4}
2.3.4.6-Tetrachlorophenol	0.96×10^{-4}
2.3.5-Trichlorophenol	1.01×10^{-4}
2.3.6-Trichlorophenol	1.01×10^{-4}
2.3.4-Trichlorophenol	1.01×10^{-4}
3.4.5-Trichlorophenol	1.01×10^{-4}
3.6-Dichloro-2.5-dihydroxy-1.4-benzoquinone	0.96×10^{-4}

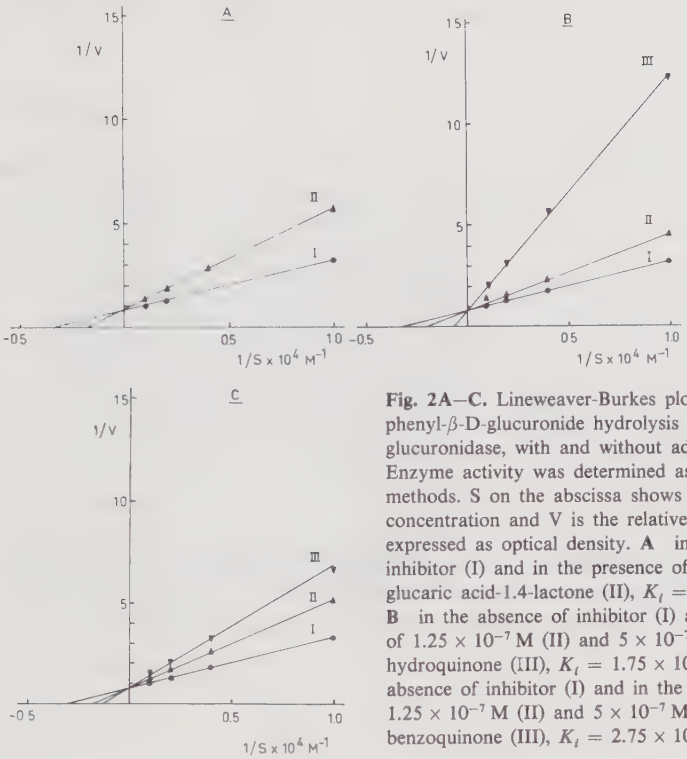

Fig. 2A—C. Lineweaver-Burkes plots of p-nitrophenyl-β-D-glucuronide hydrolysis by bacterial β-glucuronidase, with and without addition of inhibitor. Enzyme activity was determined as described in methods. S on the abscissa shows the substrate concentration and V is the relative enzyme activity expressed as optical density. **A** in the absence of inhibitor (I) and in the presence of 2.5×10^{-5} M D-glucaric acid-1.4-lactone (II), $K_i = 2.8 \times 10^{-5}$ M. **B** in the absence of inhibitor (I) and in the presence of 1.25×10^{-7} M (II) and 5×10^{-7} M tetrachloro-p-hydroquinone (III), $K_i = 1.75 \times 10^{-7}$ M. **C** in the absence of inhibitor (I) and in the presence of 1.25×10^{-7} M (II) and 5×10^{-7} M tetrachloro-p-benzoquinone (III), $K_i = 2.75 \times 10^{-7}$ M

Tetrachloro-p-hydroquinone and tetrachloro-p-benzoquinone were tested and compared with a known inhibitor of β-glucuronidase, D-glucaric acid-1.4-lactone (Niwa et al., 1972). Lineweaver-Burke plots of the inhibitory action is shown in Figure 2. The results indicate that the inhibition of bacterial β-glucuronidase by both tetrachloro-p-hydroquinone and tetrachloro-p-benzoquinone were competitive with p-nitrophenyl-β-D-glucuronide as substrate. The K_m-value of β-glucuronidase for p-nitrophenyl-β-D-glucuronide was estimated to be 3.13×10^{-4} M. The K_i-values found for tetrachloro-hydroquinone, tetrachloro-p-benzoquinone and D-glucaric acid-1.4-lactone were 1.75×10^{-7} M, 2.75×10^{-7} M and 2.8×10^{-5} M, respectively.

Inhibition of β-Glucuronidase from Liver

The tests with β-glucuronidase from bovine liver, mouse liver homogenates or rat liver microsomal preparations demonstrated that tetrachloro-p-hydroquinone and tetrachloro-p-benzoquinone had no inhibitory action on these enzymes within the pH range studied.

Figure 3 shows the β-glucuronidase activity expressed as percentage of the control activity at pH 3.8. The enzyme source used was the microsomal preparation

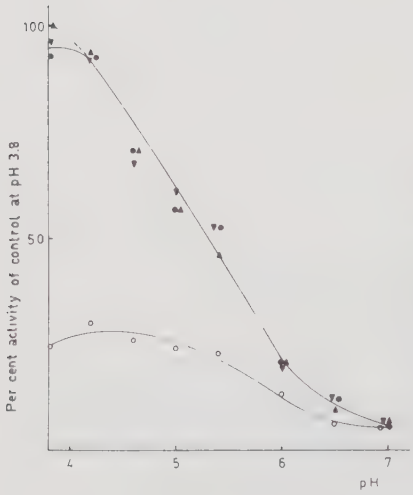

Fig. 3. The figure shows the enzyme activity expressed as percent of control at pH 3.8, with and without the addition of certain inhibitors. Enzyme source used is microsomal β-glucuronidase from rat liver prepared as described in text. Acetate buffer is used in the pH range 3.8–5.4 and phosphate buffer in the pH range 6.0–7.0. ▲—▲ with no inhibitor added; ● ● tetrachloro-p-hydroquinone 4×10^{-6}; ▼—▼ tetrachloro-p-benzoquinone 4×10^{-6}; ○—○ D-glucaric acid-1.4-lactone 5×10^{-6}

from rat liver. D-glucaric acid-1.4-lactone was included for comparison and showed a pronounced inhibitory action within the pH range 3.8–6.0. Tetrachloro-p-hydroquinone and tetrachloro-p-benzoquinone did not differ from the control where no inhibitor was used.

In vivo Inhibition of β-Glucuronidase

The in vivo test of β-glucuronidase inhibition after the administration of tetrachloro p-hydroquinone to mice was entirely negative and no inhibition could be demonstrated.

Discussion

Conjugation with glucuronic acid is a common pathway in detoxification and several compounds are excreted in urine and faeces as glucuronides. Bacterial β glucuronidase has been employed routinely in the treatment of urine in order to split conjugates of this type.

The study by Ahlborg et al. (1974) showed that tetrachloro p hydroquinone was a potent inhibitor of bacterial β glucuronidase at concentrations far below those occurring in the excreted urine from rats exposed to pentachlorophenol. This investigation clearly demonstrates that all the hydroquinones and benzoquinones studied act as potent inhibitors of the bacterial enzyme. No clear relationship to the degree of chlorination can be demonstrated and even the chlorinated pyrocatechol shows a certain inhibitory activity. The inhibition is competitive in its nature.

No effect could be demonstrated on β-glucuronidase from other sources, nor could any in vivo effect be found.

The results call for precautions when using β-glucuronidase of bacterial origin for splitting of glucuronide.

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Received January 20, 1977

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METABOLISM OF PENTACHLOROPHENOL *IN VIVO* AND *IN VITRO*

by

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ABSTRACT

Pentachlorophenol has earlier been shown to be metabolized in mammals to tetrachloro-p-hydroquinone. The metabolite possesses pronounced inhibitory activity on bacterial β-glucuronidase but not on β-glucuronidase from liver. Indirect evidence for the occurrence of both pentachlorophenol and tetrachloro-p-hydroquinone as conjugates with glucuronic acid in the urine from pentachlorophenol-treated rats is now presented. Bovine liver β-glucuronidase has been utilized to split the conjugates present.

The in vivo metabolism of pentachlorophenol has also been studied in rats treated with phenobarbital and β-diethylaminoethyldiphenyl propylacetate (SKF 525-A). In vitro metabolism has been studied using liver microsomes from rats pretreated with phenobarbital. Quantitative analysis of the compounds occurring in extracts of urine or extracts from the microsomal incubates was performed by means of mass fragmentography. Pretreatment with phenobarbital increased the metabolism of pentachlorophenol to tetrachloro-p-hydroquinone both in vivo and in vitro. SKF 525-A, however, inhibited the metabolism in vitro but enhanced the metabolism in vivo when given less frequently than every 6th hour. Dechlorination of pentachlorophenol is mediated by microsomal enzymes that can be induced by phenobarbital. SKF 525-A does not inhibit the dechlorination in vivo but does so in vitro.

Keywords

Metabolism, glucuronide, rats, microsomes, mass fragmentography, pentachlorophenol, tetrachloro-p-hydroquinone, phenobarbital, β-diethylaminoethyldiphenyl propylacetate.

ZUSAMMENFASSUNG

Wie in früheren Versuchen gezeigt werden ist, wird Pentachlorphenol bei Säugern zu Tetrachlor-p-hydrochinon metabolisiert. Dieser Metabolit wirkt ausgeprägt hemmend auf Bakterien-β-glucuronidase aber nicht auf Leber-β-glucuronidase. Ein indirektes Beweis für das Vorkommen von sowohl Pentachlorphenol als auch Tetrachlor-p-hydrochinon als Konjugate mit Glucuronsäure in Harn von Pentachlorphenolbehandelten Ratten wird nun vorgelegt. Zur Spaltung der gegenwärtigen Konjugate wurde Rinderleber-β--glucuronidase benutzt.

Der in vivo Abbau von Pentachlorphenol wurde auch an mit Phenobarbital und mit β-diaethylaminoaethyl-diphenyl propylacetat (SKF 525-A) behandelten Ratten untersucht.

Beim Studium des in vitro Abbaus wurden Lebermikrosome von mit Phenobarbital behandelten Ratten benutzt. Phenobarbital steigerte die Umwandlung in Tetrachlor-p-hydrochinon sowohl in vivo als auch in vitro. SKF 525-A verzögerte indessen den in vitro Umsatz, steigerte ihn jedoch in vivo wenn weniger oft als alle 6 Stunden gegeben. Die Untersuchung zeigt also, dass die Entchlorung von Pentachlorphenol durch Mikrosomenenzyme vermittelt wird, die durch Phenobarbital angeregt werden können. SKF 525-A hemmt die Entchlorung nicht in vivo, wohl aber in vitro.

Pentachlorophenol has been shown to be excreted in the urine from rat, mouse and man both as unchanged pentachlorophenol and as a metabolite, tetrachloro-p-hydroquinone. The two compounds were found in the urine both in conjugated and in free forms (Jacobson and Yllner, 1971; Ahlborg *et al.*, 1974). The nature of these conjugates has not been elucidated. Ahlborg *et al.*, (1974) have shown that tetrachloro-p-hydroquinone is a potent inhibitor of bacterial β-glucuronidase. Further studies (Ahlborg *et al.*, 1977) have shown that the inhibitory action of tetrachloro-p-hydroquinone could only be demonstrated for bacterial β-glucuronidase and not for β-glucuronidase from bovine liver. One purpose of the present paper is to throw further light on the nature of the conjugates by comparing the recovery of pentachlorophenol and tetrachloro-p-hydroquinone from urine of pentachlorophenol-treated rats after subjecting the urine to acid hydrolysis or incubation with bovine liver β-glucuronidase.

The mechanism of dechlorination of pentachlorophenol seems to be not very well known. The second part of the present paper represents an effort to achieve a better understanding of the dechlorination process by the use of inducing and inhibiting agents to manipulate the metabolizing enzymes responsible for dechlorination. Phenobarbital is one of several compounds known to induce the metabolism of many types of compounds (Conney, 1967) while β-diethylaminoethyldiphenyl propylacetate (SKF 525-A) has been shown to inhibit the action of several microsomal enzymes (Anders, 1971). Pentachlorophenol was therefore administered to rats subjected either to repeated pretreatment with phenobarbital or to single or repeated doses of SKF 525-A. The metabolism of pentachlorophenol was also studied *in vitro* using liver microsomes isolated from rats with and without the preceding administration of phenobarbital.

MATERIALS AND METHODS

Compounds used (abbreviations in parentheses).

β-diethylaminoethyldiphenyl propylacetate (SKF 525-A) mw 353.51. Smith, Kline and French, USA.

N,O-bis (trimethylsilyl) acetamide (BSA) mw 203.43 Pierce Chem. Co., USA.

Pentachlorophenol (PCP) mw 266.34 Chemical Manuf. Laboratory, Salinas, Calif., USA.

Phenobarbital sodium (PB) mw 254.22 Distra, England.

Tetrachloro-p-hydroquinone (tetra-CH) mw 247.89 Eastman Kodak Co., USA.

Tetrachloropyrocatechol (tetra-CPC) mw 247.89 Schuchardt, München, Germany.

Other reference compounds and chemicals of reagent grade were obtained commercially.

All animals used were male Sprague-Dawley laboratory rats, weight 150-250 g.

METHODS

1. Hydrolysis of conjugates

Rats were given PCP in propylene glycol (5 mg · ml^{-1}) in a dose of 10 mg · kg^{-1} intraperitoneally and put in all glass metabolism cages, two rats in each cage. Urine was collected in cooled vessels during 24 hours.

As an internal standard 60 µg · ml^{-1} of tetra-CPC was added to 3 ml of the collected urine which was then divided into three 1 ml portions, a, b and c, each one of which was then submitted to one of the following different treatments.

a) Acidification with concentrated hydrochloric acid to pH 2.

b) Boiling with an aliquot of concentrated hydrochloric acid for one hour in a sealed ampoule.

c) Incubation with β-glucuronidase from bovine liver (Sigma type B-1, 903.000 units · g^{-1}) as described by Sigma Chem. Co. (1973) and after that acidification with concentrated hydrochloric acid to pH 2.

The samples were then extracted with diethyl ether and derivatized with BSA to obtain trimethylsilyl ethers. Standard curves were prepared and analysis performed as described by Elkin et al., (1973).

2. Induction and inhibition of metabolizing enzymes

a) In vivo

Rats were given PB in saline in a dose of 75 mg · kg^{-1}

intraperitoneally for three consecutive days and on the fourth day PCP was administered in a dose of 10 mg · kg^{-1} intraperitoneally. Urine was collected as described earlier. Groups of 4 rats were given SKF 525-A in saline in combination with PCP (10 mg · kg^{-1}) according to Table 1. Both compounds were administered intraperitoneally.

TABLE 1. Dose scheme for the *in vivo* experiments. All doses given per kg of bodyweight.

Time	Groups			
Hours	1	2	3	4
0	SKF 525-A 25 mg	SKF 525-A 50 mg	SKF 525-A 50 mg	
1	PCP 10 mg	PCP 10 mg	PCP 10 mg	PCP 10 mg
6			SKF 525-A 50 mg	
8		SKF 525-A 50 mg		
12			SKF 525-A 50 mg	
18			SKF 525-A 50 mg	
25	urine collected	urine collected	urine collected	urine collected
49			urine collected	urine collected

Urine was collected as described earlier with the addition of 48 hour samples from groups 3 and 4. During the last 24 hours no compound was administered. The urine was boiled with concentrated hydrochloric acid, extracted and analysed as described earlier.

b) In vitro

Microsomes were prepared essentially as described by Ernster et al., (1962) from the livers of male Sprague-Dawley rats (150-250 g) with and without pretreatment with PB for 5 consecutive days (75 mg/kg i.p.). The rats were starved overnight and killed by decapitation by guillotine and exsanguinated. The livers were removed and weighed in 0.25 M sucrose. A 25% crude homogenate in 0.25 M sucrose was prepared by homogenizing the minced tissue in a glass Potter-Elvehjem homogenizer. The homogenate was centrifuged in an MSE superspeed 50 centrifuge at 500 x g for 2 minutes and at 13.300 x g for 8 minutes. The pellet was discarded and the supernatant centrifuged at 105.000 x g for 60 minutes. The pellet was then suspended in 0.15 M KCl and recentrifuged at 105.000 x g for 30 minutes. The new pellet was suspended in 0.25 M sucrose to a final concentration of 0.5 g liver/ml. All procedures were carried out at 0° - 4°C. Microsomal protein was determined as described by Lowry et al., (1951) with the modifications recommended by Miller, (1959).

PCP was dissolved in acetone and added to the incubation vessel. Acetone was evaporated and the NADPH-generating system was added. To some incubates SKF 525-A was used in a concentration of 5×10^{-4} M, and the vessels were pre-incubated for 1-2 minutes. The reaction was started with microsomes, and the mixture was incubated at 37°C for 20 minutes. The incubation mixture contained in a final volume of 200 µl:

62.5 mM TRIS-HCl buffer
6.25 mM $MgCl_2$
6.25 mM Isocitric acid
1.25 mM $NADP^+$, and isocitric dehydrogenase enough to reduce 0.64 µmoles $NADP^+$ per min
5 µg of PCP
0.35-0.9 mg of microsomal protein

Microsomal protein could not be determined prior to the incubation and the later determinations showed a variation within the range indicated above. The reaction was stopped with 200 µl of 15% TCA and 2,3,4,5-tetrachlorophenol as an internal standard was added. The precipitate was removed by centrifugation for 5 minutes at 2500 rpm.

The precipitates were washed with 2 x 200 µl of methanol and the supernatants were pooled. 4 ml of distilled water was added and the samples were extracted with diethyl ether 1 x 2 ml and 2 x 1 ml.

The ether phases were removed, pooled and evaporated in N_2-flow. The samples were derivatized with diazoethane (15'), evaporated to dryness and dissolved in 0.5 ml hexane and washed with an aliquot of 20% methanol in water according to Cranmer and Freal, (1970). Hexane was evaporated and the residues were dissolved in hexane to a final volume of 100 µl which was submitted to analysis.

ANALYSIS

The analytical work was done with gas chromatography - mass spectrometry performed on an LKB model 9000 gas chromatography - mass spectrometer (LKB-Produkter, Bromma, Sweden). The separations were made on silanized glass columns (1.6 m x 3 mm i.d.) packed with 3% SE-52 on Gas Chrom Q 100-120 mesh

and maintained at a temperature of 160°C. The flow rate of helium carrier gas was 25 ml/min. The ionizing potential and trap current were 50 eV and 60 μA, respectively. The temperature of the flash heater was 210°C and the ion source was kept at 250°C. For the mass fragmentographic analysis the instrument was run under computer control as described by Elkin et al., (1973).

RESULTS

1. Hydrolysis of conjugates

The extraction results after different treatments of the urine varied and standard curves were thus prepared for each of the three procedures. The urinary levels of PCP and tetra-CH after the varying treatments of the urine are given in Table 2. Both types of hydrolytic treatment increased the recovery of free PCP and tetra-CH and the total increase was about 50%. The major part of this increase was due to a three to four-fold increase in the tetra-CH recovery. The percentual distribution of the excreted compounds in the urine was PCP 60%, PCP-glucuronide 9-16%, tetra-CH 7% and tetra-CH-glucuronide 16-22%.

2. Induction and inhibition of metabolizing enzymes

a) In vivo

After analysis of the urine samples the levels of PCP and tetra-CH were calculated from the standard curves and the ratios tetra-CH/PCP were determined. These ratios are given in Fig. 1. During the first 24 hours there was an almost 6-fold increase in the conversion of PCP to tetra-CH in the animals pretreated with PB. The administration of SKF 525-A once or twice also increased the metabolism 3-fold and 2-fold respectively while the repeated administration of SKF 525-A every 6th hour resulted in an unchanged ratio of tetra-CH/PCP as compared to the untreated controls. During the second 24 hours the conversion of PCP to tetra-CH was decreased as compared to controls.

TABLE 2. Levels of PCP and tetra-CH in urine from rats administered PCP intraperitoneally (10 mg · kg^{-1}). Urine collected during 24 hours.

Urine treatment	PCP	tetra-CH	PCP + tetra-CH	PCP + tetra-CH expressed as PCP
	µg/ml	µg/ml	µg/ml	
Not hydrolyzed urine	72.0	8.0	80.0	80.6
Hydrolyzed with hydrochloric acid	91.5	27.1	118.6	120.6
Hydrolyzed with β-glucuronidase	83.2	34.4	117.6	121.2

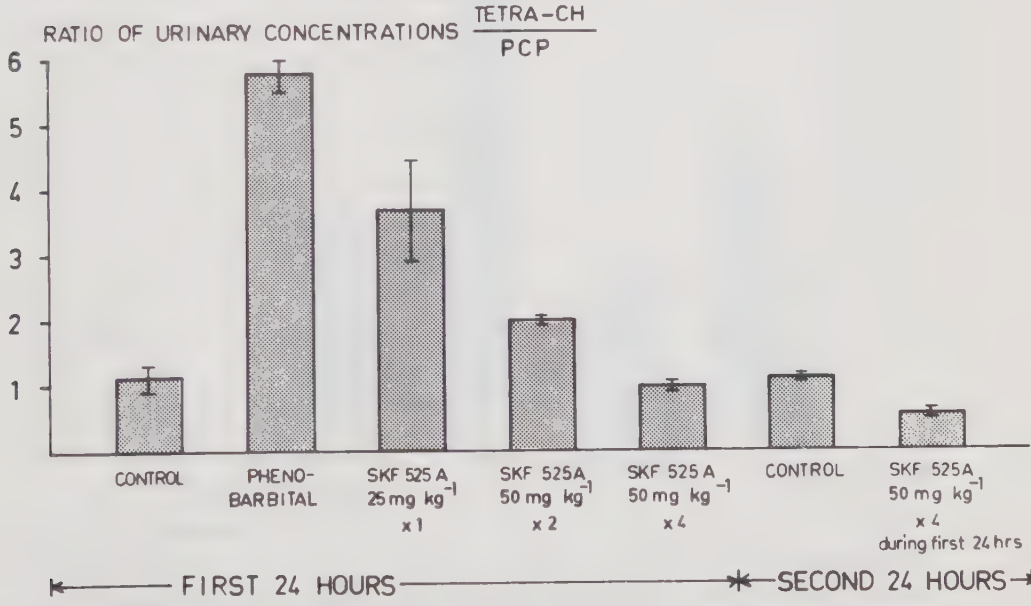

Fig. 1. Ratios of tetrachloro-p-hydroquinone versus pentachlorophenol in urine from rats administered pentachlorophenol intraperitoneally (10 mg · kg^{-1}) under various conditions as described in Table 1.

b) In vitro

The percent tetra-CH produced in the different microsomal incubations are given in Fig. 2. The use of liver microsomes from rats pretreated with PB increased the metabolizing activity of the system about 3-fold and the presence of SKF 525-A resulted in a pronounced inhibition of the reaction both when noninduced and induced microsomes were used.

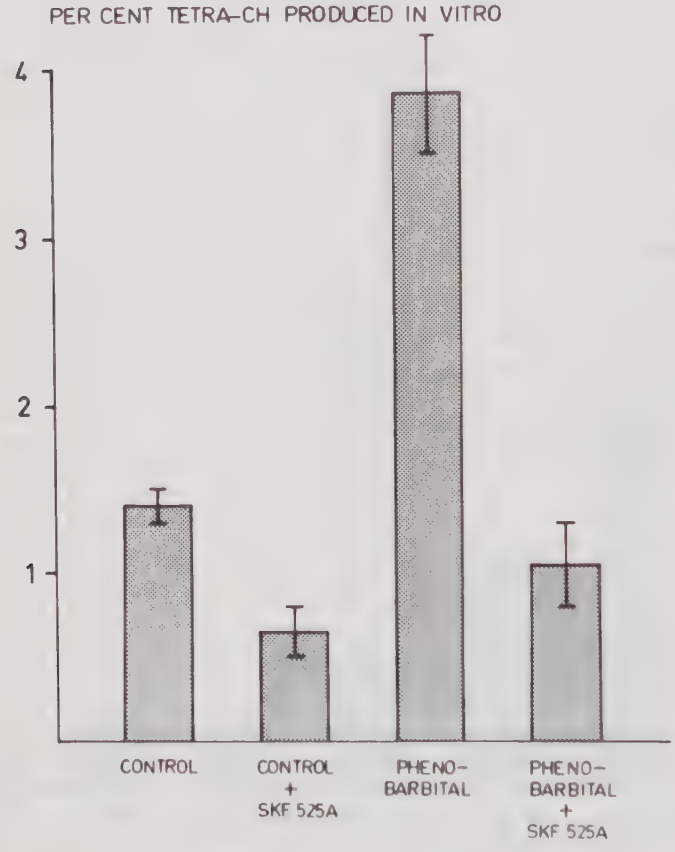

Fig. 2. Percentual metabolism of pentachlorophenol to tetrachloro-p-hydroquinone in vitro using liver microsomes from rats with and without pretreatment with phenobarbital and with and without the addition of SKF 525-A ($5 \cdot 10^{-4}$ M) to the incubates. Incubation time 20'.

DISCUSSION

In the first part of the present study it has been shown that hydrolytic treatment of the urine from PCP-treated rats, either by boiling with concentrated hydrochloric acid or by incubation with ß-glucuronidase from bovine liver, increases the amount of extractable PCP and tetra-CH with about 50%. This is indirect evidence for the presence of both compounds partly as conjugates with glucuronic acid.

Recently the presence of PCP-glucuronide in urine from PCP exposed rats has been reported by Braun and Sauerhoff, (1976). No documentation on its identification is however available. The amount of PCP-glucuronide in urine was reported to be 9.4% which is in the same range as found by us (9-16%). By means of gas chromatography - mass spectrometry, Braun and Sauerhoff (1976) also identified tetra-CH. This metabolite was reported to occur only in unconjugated form. Our data, however, clearly indicate that the greater part of the tetra-CH is present as conjugate with glucuronic acid since both types of hydrolytic treatments increase the recovery of tetra-CH three to four times. In the monkey, however, PCP seems to be excreted essentially unchanged and unconjugated (Braun and Sauerhoff, 1976).

As could be expected, the pretreatment of the rats with PB resulted in a pronounced increase in the in vivo conversion of PCP to tetra-CH. The effect of pretreatment with SKF 525-A would supposedly be an inhibition of this metabolic conversion. The results of a single administration, however, show an increase, though not as pronounced as in the phenobarbital induced animals. The lack of inhibition may be due to the short action of SKF 525-A but this does not explain the increase observed. Serrone and Fujimoto, (1962) have reported a biphasic effect of SKF 525-A. When given orally as pretreatment to mice in a dose of 20 mg/kg there was an increase in hexobarbital sleeping time after pretreatment with intervals of 1 to 6 hours. When given 16-72 hours before

hexobarbital, the effect was a small decrease in sleeping time, indicating a more rapid metabolism of hexobarbital. Kato *et al.*, (1964) reported in rats an increase of about 50% in the metabolism of pentobarbital, hexobarbital, meprobamate, carisprodol and strychnine 48 hours after administration of SKF 525-A. The induction of metabolizing enzymes has also been shown to increase the metabolism of SKF 525-A itself, (Anders and Mannering, 1966).

In our own experiments the ratio tetra-CH/PCP in the rats given SKF 525-A twice is somewhat lower than the ratio after one single pretreatment indicating a rather short duration of the inhibiting effect of SKF 525-A and supporting the explanation that a second phase of stimulated metabolism of the administered PCP occurs.

When SKF 525-A is given every sixth hour no stimulation of the PCP metabolism *in vivo* can be demonstrated, neither can any inhibition be seen during the first 24 hours. This could be explained either by an insignificant inhibitory action of SKF 525-A on the enzymes responsible for the dechlorination of PCP or by a shortlasting effect. The data from the *in vitro* experiments, however, clearly show that SKF 525-A does inhibit the conversion of PCP to tetra-CH. The lack of inhibition *in vivo* might thus be due to the fact that the enhancement is more pronounced than the inhibition when taken together during the time interval studied.

The action of SKF 525-A is thus biphasic with an initial strong inhibiting action followed by a second phase of stimulation. The data reported here indicate that with regard to PCP metabolism the stimulatory phase starts within the first 24 hours and that it can be abolished by repeated administration of SKF 525-A every 6th hour. After such treatment no stimulatory effect appeared during the 24 hours following the last administration of SKF 525-A.

The decrease in metabolism during the second 24 hours is more difficult to understand and more data will be necessary to explain this.

There seems to be no indication in the literature that the inhibitory effect of SKF 525-A turns into enhancement with a change in dose. It is therefore less likely that a possible difference in concentrations of SKF 525-A *in vitro* and *in vivo* can explain the different effects noted.

SKF 525-A itself is degraded with the formation of several metabolites that retain the inhibiting properties (Anders and Mannering, 1966; Anders *et al.*, 1966). The inhibitory action of the metabolites, however, seems to be more shortlasting than that of the parent compound (Stitzel *et al.*, 1966) and an effect of the metabolites can thus not explain the inhibition seen during the time period 25-49 hours when no SKF 525-A was administered.

Compared to the *in vivo* experiments the *in vitro* data shows a rather low rate of transformation of PCP to tetra-CH. This is mainly due to the short incubation time (20 min) but may also depend on the incubation being a closed system that cannot dispose of the products formed. The greater part of the excreted tetra-CH is conjugated with glucuronic acid and the glucuronidation of tetra-CH could be the rate limiting factor as far as excretion is concerned.

The dechlorination of PCP to tetra-CH is thus mediated by microsomal enzymes that can be induced by PB. The transformation can be inhibited *in vitro* by SKF 525-A but is not very pronounced *in vivo*. The stimulating effect of SKF 525-A on the *in vivo* metabolism of PCP seems to be more pronounced than for the compounds studied by Kato *et al.*, (1964), i.e. more than 200 percent for PCP versus about 50 percent for the latter compounds. The poor inhibitory effect of SKF 525-A and the accompanying enhancement of dechlorination *in vivo* constitute observations that may aid further studies of metabolism.

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ACKNOWLEDGMENT

This work was supported by grants from the Swedish Environment Protection Board (7-1/76 and PK-345/76).

EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN ON THE *IN VIVO* AND *IN VITRO* DECHLORINATION OF PENTACHLOROPHENOL

by

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ABSTRACT

The metabolism of pentachlorophenol has been studied in the rat after pretreatments with phenobarbital, 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In addition to the previously identified metabolite, tetrachloro-p-hydroquinone, trichloro-p-hydroquinone has been identified in urine as a metabolite. The formation of the latter represents a type of dechlorination different from that of the formation of tetrachlorohydroquinone. The inducing agents, 3-methylcholanthrene and TCDD have similar effects on the dechlorination and increase the formation of tetrachloro-p-hydroquinone more pronounced than does phenobarbital. In contrast to phenobarbital they also increase the formation of trichloro-p-hydroquinone and the total elimination of pentachlorophenol and its metabolites. The *in vivo* findings are supported by *in vitro* studies with microsomes from rats pretreated with phenobarbital or TCDD. Use of the inhibitor β-diethylaminoethyl-diphenyl propylacetate (SKF 525-A) *in vitro* showed a more pronounced inhibition on microsomes from phenobarbital-treated rats than on microsomes from untreated or TCDD-treated rats.

Gas chromatography - mass spectrometry have been used for the identification and quantification of pentachlorophenol and its metabolites.

Keywords

Metabolism, dechlorination, urinary excretion, rats, microsomes, pentachlorophenol, tetrachloro-p-hydroquinone, trichloro-p-hydroquinone, 2,3,7,8-tetrachlorodibenzo-p-dioxin, β-diethylaminoethyl-diphenyl propylacetate, phenobarbital, 3-methylcholanthrene.

ZUSAMMENFASSUNG

Der Metabolismus von Pentachlorphenol nach Vorbehandlung der Versuchstiere (Ratten) mit Phenobarbital, 3-Methylcholantren oder 2,3,7,8-Tetrachlordibenzo-p-dioxin (TCDD) ist untersucht worden. Zu dem schon früher nachgewiesenen Metaboliten Tetrachlor-p-hydrochinon wurde nun auch Trichlor-p-hydrochinon als Harnmetabolit festgestellt. Die Bildung des Letzteren stellt eine andere Art von Entchlorung dar als die jenige die bei der Entstehung von Tetrachlor-p-hydrochinon vorliegt. 3-Methylcholantren und TCDD haben ähnlicher Einfluss auf die Entchlorung und steigern die Bildung von Tetrachlor-p-hydrochinon mehr ausgeprägt als es bei Phenobarbital der Fall ist. In Kontrast zu Phenobarbital steigern sie auch die Bildung von Trichlor-p--hydrochinon sowie die totale Eliminierung von Pentachlorphenol und den Metaboliten. Die in Vivo Funde werden von in Vitro Studien mit Mikrosomen von mit Phenobarbital oder TCDD vorbehandelten Ratten unterstützt. Anwendung des Inhibitors β-Diethylaminoethyl-diphenyl propylacetat (SKF 525-A) zeigte in Vitro eine ausgeprägtere Inhibition auf Mikrosome von mit Phenobarbital behandelten Ratten als auf Mikrosome von unbehandelten oder TCDD-behandelten Ratten. Nachweis und Bestimmung von Pentachlorphenol und seinen Metaboliten wurden gaschromatografisch-massenspektrometrisch durchgeführt.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) may occur as a contaminant in the production of 2,4,5-trichlorophenol from tetrachlorobenzene. The 2,4,5-trichlorophenol is subsequently used for the synthesis of the herbicide 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and the antiseptic hexachlorophene. The occurrence of TCDD as a contaminant in 2,4,5-T has caused great concern because of the wide spectrum of toxic effects displayed by TCDD. One of the main features of TCDD is its ability to influence certain enzyme systems when animals are exposed to sublethal doses of TCDD. Hepatic microsomal mixed function oxidases seem to be particularly sensitive (Greig and De Matteis, 1973; Lucier et al., 1973).

Poland and Glover (1973; 1974) have shown that TCDD is a potent inducer of aryl hydrocarbon hydroxylase and that TCDD is more potent than any other inducer on a dose per unit body weight basis. In a comparison between TCDD and 3-methylcholanthrene (3-MC), it was found that the maximal induction that can be achieved was the same for the two compounds, the effect of TCDD was, however, considerably more longlasting.

In mammals pentachlorophenol (PCP) has been shown to undergo dechlorination with the subsequent formation of tetrachloro-p-hydroquinone (tetra-CH) (Jacobson and Yllner, 1971; Ahlborg et al., 1974). The dechlorination of PCP can be enhanced in rats by pretreatment with phenobarbital (PB) (Ahlborg et al., 1977). Dechlorination seems to be an important step in the detoxification of many chlorinated compounds e.g. PCP to tetra-CH, hexachlorobenzene to PCP and tetra-CH (Koss et al., 1976), lindane to tetra- and trichlorophenols (Chadwick and Freal, 1972), but the mechanisms behind are poorly understood.

Since TCDD has been shown to increase several types of biodegradations it was considered worthwhile to explore its

actions with regard to dechlorination. The present study was thus undertaken in order to compare the effects of TCDD and other inducers of enzyme systems on the dechlorination of PCP.

MATERIALS AND METHODS

Reference compounds and other chemicals were obtained from various sources according to the following list.

Abbreviations used in text are given in parenthesis.

β-Diethylaminoethyl-diphenyl propylacetate (SKF 525-A), mw 353.51, Smith, Kline and French, U.S.A.

Pentachlorophenol (PCP), purity 99.9%, mw 266.34, Chemical Manuf. Laboratory, Salinas, California, U.S.A.

Tetrachloro-p-hydroquinone (tetra-CH), mw 247.89, Eastman Kodak Co., U.S.A.

Phenobarbital sodium (PB), mw 254.22, Distra, England.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), mw 321.99, a gift from DOW Chem. Co., U.S.A.

3-Methylcholanthrene (3-MC), mw 268.34, Sigma Chemical Co., U.S.A.

Trichloro-p-hydroquinone (tri-CH), mw 213.46, was synthesized as described by Ahlborg and Larsson (1977).

Other reference compounds and chemicals of reagent grade were obtained commercially.

All animal experiments were performed with male Sprague-Dawley rats (weight 150-250 g).

METHODS

a) In vivo

Prior to the administration of PCP the rats were pretreated with different compounds according to the following:

TCDD was given in corn oil in a dose of 10 μg · kg^{-1} (31 nM · kg^{-1}) by gavage 4 days before exposure to PCP. 3-MC was given in corn oil, 20 mg · kg^{-1} (75 μM · kg^{-1}) intraperitoneally 4 days before exposure and PB was given in saline in a dose of 75 mg · kg^{-1} (295 μM · kg^{-1}) intraperitoneally daily for 5 days before exposure. Controls were given corn oil, 2 ml · kg^{-1}, by gavage 4 days before exposure.

PCP was administered in propylene glycol in a dose of 10 mg · kg^{-1} (38 μM · kg^{-1}) intraperitoneally and the animals were placed in all glass metabolism cages, 2 animals in each cage. Urine was collected every 24 hours in a cooled vessel during 3 days. Extractions of urine and analysis were performed as described previously (Ahlborg et al., 1977). In addition, trichloro-p-hydroquinone was included in the quantitative analyses.

b) In vitro

The rats were pretreated as described above, but they were fasted over night before the experiment. The rats were killed by decapitation, the livers were removed and microsome preparations, incubations and analysis were performed as described by Ahlborg et al. (1977). All incubations were done both with and without the addition of SKF 525-A at a concentration of 5×10^{-4} M. No incubations with microsomes from 3-MC-pretreated rats were performed.

RESULTS

a) In vivo

By means of gas chromatography - mass spectrometry, tri-CH was identified as a urinary metabolite of PCP in addition to the earlier identified metabolite tetra-CH.
The total amounts of PCP (in mg) excreted during the first 24 hours and of the metabolites tetra-CH and tri-CH under the various pretreatment conditions are given in Fig. 1.

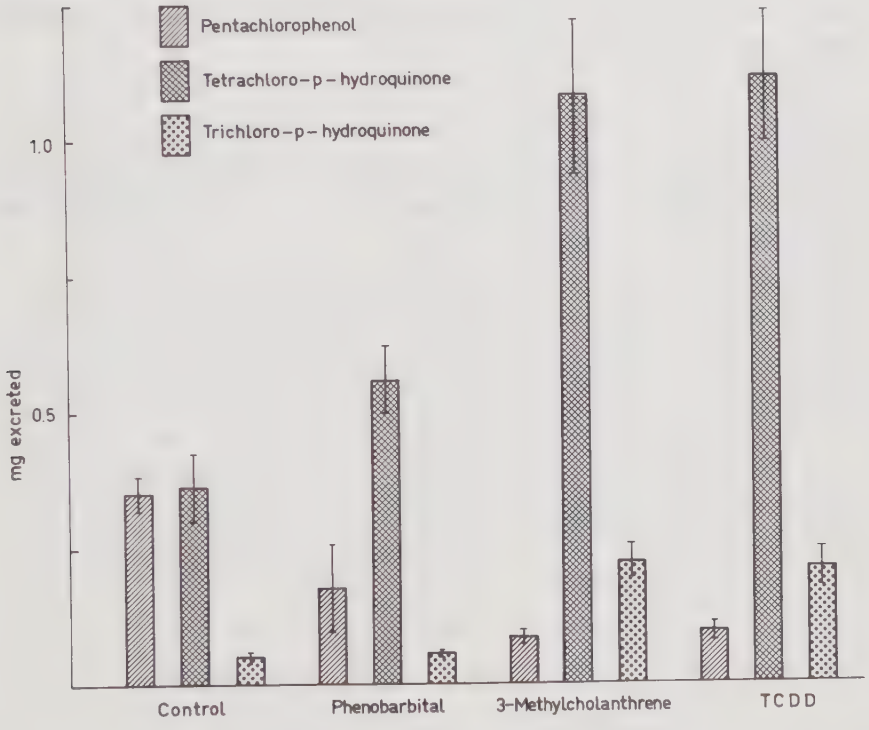

Fig. 1. Total amounts of PCP, tetra-CH and tri-CH excreted in the urine during the first 24 hours after intraperitoneal administration of PCP 10 mg · kg^{-1} to rats pretreated with the different compounds indicated in the figure. Values are means of 4 rats with total deviation indicated.

Pretreatment with PB increased the amount of tetra-CH excreted but had no influence on the amount of tri-CH. Pretreatment with 3-MC and TCDD increased the amount of tetra-CH excreted more pronounced but did also increase the amount of tri-CH.

The urinary recoveries of unchanged PCP, tetra-CH and tri-CH are given in Tables 1 and 2, where all the values have been transformed to PCP-equivalents. No difference with regard to total recovery can be shown between the control rats and the rats pretreated with PB while both groups of rats pretreated with 3-MC or TCDD show an increased total output, essentially during the first 24 hours.

TABLE 1. Daily urinary excretion of PCP, tetra-CH and tri-CH during 72 hours after the administration of PCP 10 mg · kg^{-1} intraperitoneally. All values are calculated as PCP-equivalents and given in µg.

Pretreatment	Compound	Day 1	Day 2	Day 3
Control	PCP	351	40	8
	tetra-CH	391	184	48
	tri-CH	60	-	-
PB	PCP	175	23	-
	tetra-CH	600	121	19
	tri-CH	69	-	-
3-MC	PCP	134	3	-
	tetra-CH	1160	105	11
	tri-CH	280	-	-
TCDD	PCP	92	3	-
	tetra-CH	1199	102	15
	tri-CH	262	-	-

TABLE 2. Summarized urinary excretion of PCP, tetra-CH and tri-CH following single intraperitoneal dose of PCP (10 mg · kg^{-1}) after different pretreatments as described in the text. All values are expressed as percent of the given dose.

Pretreatment	Day 1	Day 2	Day 3	Total
Control	36.6	10.2	2.5	49.3
PB	40.6	6.9	0.9	48.4
3-MC	74.6	5.1	0.5	80.2
TCDD	75.0	5.1	1.0	81.1

b) In vitro

The percentual metabolism of PCP to tetra-CH is demonstrated in Fig. 2. The metabolic activity was increased almost 3-fold when microsomes from PB pretreated rats were used as compared to the control while the increase was more than 5-fold when microsomes from TCDD-pretreated rats were used. The effect of SKF 525-A was more pronounced when microsomes from PB-pretreated rats were used than when microsomes from untreated and TCDD-pretreated rats were used.

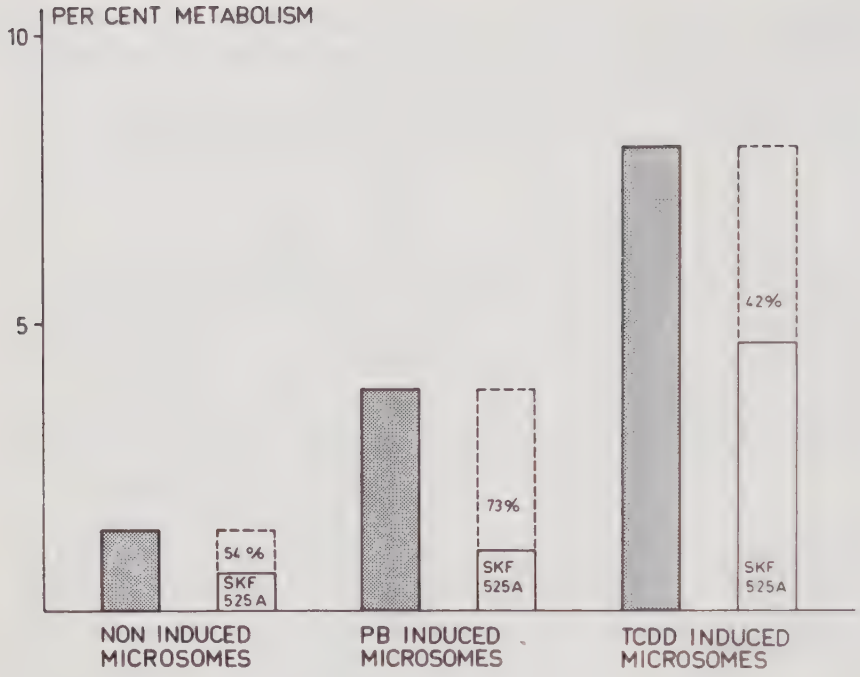

Fig. 2. Percentual metabolism of PCP to tetra-CH in vitro using liver microsomes from untreated rats or from rats pretreated with PB or TCDD respectively. Incubation time 20'.

Figures in the bars indicate the percentual inhibition of SKF 525-A (5×10^{-4} M).

DISCUSSION

In a previous report (Ahlborg et al., 1974) tri-CH was tentatively identified in the urine from PCP-exposed rats. The occurrence was interpreted as depending on tetrachlorophenol impurities in the PCP used. The PCP used in this study, however, has a purity of 99.9% and the fact that the amounts of tri-CH formed are related to different inducing pretreatments can only be explained as the result of a further dechlorination of PCP. Tri-CH has also been identified as a minor urinary metabolite in rats exposed to 2,3,4,5- or 2,3,4,6-tetrachlorophenol (Ahlborg and Larsson, 1977).

The identification of tri-CH as a metabolite of PCP means that two different types of dechlorination are taking place, one where chlorine is replaced by a hydroxygroup and a second one where chlorine is replaced by hydrogen. This might be effected by different enzyme systems as indicated by the effect of different pretreatments where the formation of tri-CH is enhanced in animals pretreated with 3-MC or TCDD but not in animals pretreated with PB.

The formation of tetra-CH, however, is much more enhanced by the pretreatment with TCDD or 3-MC than with PB and the higher concentration of tetra-CH may thus explain at least part of the increase of tri-CH seen.

If the formation of tetra-CH would be the result of a two step reaction, i.e. dechlorination followed by a hydroxylation it should be possible to identify 2,3,5,6-tetrachlorophenol as an intermediary metabolite. The occurrence of tetrachlorophenol as a urinary metabolite of pentachlorophenol has been described by Engst et al. (1976), but we have not been able to verify their findings mass spectrometrically. Furthermore, 2,3,5,6-tetrachlorophenol is rather stable and only metabolized to and excreted as tetra-CH to about 35 percent (Ahlborg and Larsson, 1977).

Our data indicate that there is a difference in the effects of PB as compared to the effects of TCDD or 3-MC. This is further supported by the data given in Table 2, which indicate that pretreatment with TCDD or 3-MC increases the elimination of PCP and its metabolites while there is no such effect after pretreatment with PB.

The steps involved in the elimination of PCP thus include two types of dechlorination and partial glucuronidation of PCP and tetra-CH (Ahlborg et al., 1977). Most probably tri-CH will also form a conjugate, though this has not been studied in the present work.

It is well known that there is a difference in effect between PB and 3-MC with regard to induction of certain cytochromes, cytochrome P-450 being induced by PB and cytochrome P_1-450 (P-448) by 3-MC (Mannering, 1971). TCDD also induces P_1-450 and is in several respects similar to 3-MC with regard to effects on microsomal oxygenases, the difference being mainly in potency and duration of action (Poland and Glover, 1974). This is in agreement with our findings where all our in vivo data indicate identical effects of 3-MC and TCDD.

The results of our in vitro studies indicate differences between PB and TCDD that are in good agreement with the in vivo findings. Due to the low percentage of metabolites formed during the short incubation time (20 minutes) the formation of tri-CH could, however, not be studied.
SKF 525-A was incorporated in the in vitro studies and showed a pronounced inhibition on all types of microsomes studied. The inhibition was, however, most pronounced with microsomes from rats pretreated with PB, while no significant difference with regard to inhibition could be seen when noninduced microsomes and microsomes from TCDD-pretreated animals were compared. These results further support the view that different mechanisms are affected by PB and TCDD respectively.

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ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Environment Protection Board (7-1/76 and PK-345/76).

METABOLISM OF TETRACHLOROPHENOLS IN THE RAT

by

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ABSTRACT

The three isomers of tetrachlorophenol were administrated intraperitoneally to rats and the urinary excretion products studied. Tetrachloro-p-hydroquinone was identified as a major metabolite of 2,3,5,6-tetrachlorophenol, constituing about 35% of the dose given. Trichloro-p-hydroquinone was identified as a minor metabolite of both 2,3,4,5- and 2,3,4,6-tetrachlorophenol. 2,3,5,6-tetrachlorophenol was eliminated within 24 hours, 2,3,4,6--tetrachlorophenol within 48 hours while only 60% of the given dose of 2,3,4,5-tetrachlorophenol could be recovered within 72 hours.

The acute toxicity of the tetrachlorophenols and tetrachloro-p--hydroquinone was studied in mice upon oral and intraperitoneal administration. 2,3,5,6-tetrachlorophenol (LD_{50} p.o. 109 mg $\cdot$ kg^{-1}) was the most toxic compound followed by 2,3,4,6- and 2,3,4,5--tetrachlorophenol (LD_{50} p.o. 131 and 400 mg $\cdot$ kg^{-1}, respectively). Tetrachloro-p-hydroquinone proved to have low oral toxicity (LD_{50} p.o. 500 mg $\cdot$ kg^{-1}) but was more toxic than the tetrachlorophenols when administered intraperitoneally. The oral LD_{50} for pentachlorophenol, under identical experimental conditions, was found to be 74 mg $\cdot$ kg^{-1}.

Keywords

Metabolism, urinary excretion, rats, toxicity, mice, tetrachlorophenol, tetrachloro-p-hydroquinone, trichloro-p-hydroquinone, tetrachloropyrocatechol, pentachlorophenol.

ZUSAMMENFASSUNG

Die drei Isomeren von Tetrachlorphenol wurden an Ratten intraperitoneal verabreicht und die Ausscheidungsprodukte in Harn untersucht. Tetrachlor-p-hydrochinon erwies sich als Hauptmetabolit von 2,3,5,6-Tetrachlorphenol, entsprechend etwa 35% der zugeführten Menge. Bei sowohl 2,3,4,5- als auch 2,3,4,6-Tetrachlorphenol wurde Trichlor-p-hydrochinon als Metabolit festgestellt, jedoch in geringer Menge. Das 2,3,5,6--Tetrachlorphenol wurde innerhalb von 24 Stunden und das 2,3,4,6-Tetrachlorphenol innerhalb von 48 Stunden eliminiert während bei 2,3,4,5-Tetrachlorphenol innerhalb von 72 Stunden nur 60% der gegebenen Menge erfasst werden konnte.

Die akute Toxicität von Tetrachlorphenolen und Tetrachlor-p--hydrochinon wurden an Mäusen per oral sowie intraperitoneal studiert. 2,3,5,6-Tetrachlorphenol erwies sich als die toxischste der Substanzen (LD_{50} p.o. 109 mg · kg^{-1}), gefolgt von 2,3,4,6- und 2,3,4,5-Tetrachlorphenol (LD_{50} p.o. 131 mg · kg^{-1} und 400 mg · kg^{-1}). Tetrachlor-p-hydrochinon zeigte nur geringe Toxicität, wenn oral verabreicht (LD_{50} 500 mg · kg^{-1}), war aber toxischer als die Tetrachlorphenole, wenn intraperitoneal gegeben.

Tetrachlorophenols are in use as fungicides in different applications (i.e. wood preservation) and they have also been reported as mammalian metabolites of the γ-isomer of benzen hexachloride (lindane) in rats (Chadwick and Freal, 1972) and rabbits (Karapally et al., 1973). Tetrachlorophenols also constitute a major impurity in many commercial pentachlorophenol preparations.

Available information on the toxicity of tetrachlorophenols is very limited. In a study by Farquharson et al. (1958) they found the median lethal dose of 2,3,4,6-tetrachlorophenol given intraperitoneally in olive oil to rats to be 130 mg/kg as compared to 56 mg/kg for pentachlorophenol and 276-372 mg/kg for four different isomers of trichlorophenol. The acute toxicity thus seems to increase with an increase in chlorination. The mode of action of tetrachlorophenols (e.g. uncoupling of oxidative phosphorylation) seems to be the same as for pentachlorophenol but at least in in vitro experiment the effects are less pronounced than for pentachlorophenol (Weinbach and Garbus, 1965). No information has been available hitherto concerning the metabolism of tetrachlorophenols.

Recent studies of pentachlorophenol have shown that that compound, contrary to what has been reported earlier, is metabolized in mammals (Tashiro et al., 1970; Jacobson and Yllner, 1971; Elkin et al., 1973; Ahlborg et al., 1974; Braun and Sauerhoff, 1976).

Lack of sufficient information has prompted a study of the metabolism and urinary excretion of the three different isomers of tetrachlorophenol in the rat as well as a determination of the acute toxicity of the compounds.

MATERIAL AND METHODS

1. Reference compounds

Tetra- and trichlorophenols were obtained from commercial sources according to the following list:

2,3,4,5-tetrachlorophenol from Aldrich Chem., USA or Fluka AG, Switzerland.
2,3,5,6-tetrachlorophenol from Aldrich Chem., USA or Fluka AG, Switzerland.
2,3,4,6-tetrachlorophenol from Fluka AG, Switzerland.
Tetrachloropyrocatechol from Schuchardt, Germany.
Tetrachloro-p-hydroquinone from Eastman Kodak Co., USA.

2,3,5-trichloro-p-hydroquinone was synthetized from 2,6-dichloro--p-benzoquinone (Eastman Kodak Co., USA) by the route given by Oliverio (1948) as specified below.

1.8 g 2,6-dichloro-p-benzoquinone was dissolved in 15 ml acetylchloride, 5 g aluminiumchloride was added in small portions and the mixture was left standing in a closed vessel until a white compound precipitated (2-4 hours). The mixture was moved to a beaker with ice and 50 ml 3 M hydrochloric acid. The precipitate was isolated by filtering and recrystallized in methanol. The resulting compound, 2,3,5-trichloro-p-hydroquinone diacetate (m.p. 153^{o}C), was then boiled with 10% (v/v) sulfuric acid in which the compound dissolved very slowly (>36 hours). After solubilization the product was allowed to crystallize and it was then suctionfiltered while cooled to 0^{o}C. The resulting 2,3,5--trichloro-p-hydroquinone had a m.p. of 132^{o}C and the identity and purity was checked by means of gas chromatography - mass spectrometry.

All the isomers of tetrachlorophenol were checked for impurities by gas chromatography - mass spectrometry in order to avoid

contamination with other chlorinated phenols that could give erroneous results in the determination of the excretion products. 2,3,4,5- and 2,3,5,6-tetrachlorophenol were found to be free from such impurities while 2,3,4,6-tetrachlorophenol was found to contain about 20% pentachlorophenol. In order to purify the latter compound 500 mg of the available technical 2,3,4,6-tetrachlorophenol was dissolved in methanol and run through an ion exchange column (Dowex 2 x 8, column 13 x 200 mm) with gradient elution as described by Skelly (1961). After elution, the samples containing tetrachlorophenol (as determined by spectrophotometry) were pooled. The methanol was evaporated in a Rotavapor (Büchi, Switzerland) and distilled water was added to the acetic acid that remained, with the precipitation of the compound as a result. The diluted acetic acid was shaken with diethyl ether, the ether phase was saved and the ether was evaporated with a gentle nitrogen stream. The residue was repeatedly washed with distilled water and centrifuged in order to remove remaining acetic acid. The cleaned residue was dissolved in diethyl ether and evaporated to dryness. 1 mg was dissolved in 0.3 ml diethyl ether and 0.2 ml N,O-bis (trimethylsilyl) acetamide (BSA) and subjected to mass fragmentography for control of the purity.

Control of the purity of tetrachloropyrocatechol revealed the occurrence of two major impurities and one minor, all together constituting about 15%. Judging from mass spectra the two major impurities are two different isomers of trichloropyrocatechol while the minor impurity probably is a dichloropyrocatechol. The compound was subjected to similar treatment of purification as 2,3,4,6-tetrachlorophenol.

Other reference compounds and chemicals of reagent grade were obtained commercially.

Abbreviations used:

PCP: pentachlorophenol

tetra-CP: tetrachlorophenol

tetra-CPC: tetrachloropyrocatechol
tetra-CH: tetrachloro-p-hydroquinone
tri-CH: trichloro-p-hydroquinone
TMS: trimethylsilyl ether

2. Animal experiments

a) Metabolism studies

Rats (Spraque Dawley, 200-300 g) were given the respective chlorophenols (10 mg · kg^{-1}), dissolved in propylene glycol (5 mg · ml^{-1}), intraperitoneally. After administration, the animals were placed in glass metabolism cages, 2 rats in each cage, designed for the separate collection of urine and faeces.

The urine was collected in a cooled vessel continuously flushed with nitrogen and urine samples were taken at 24, 48 and 72 hours. The animals were allowed water and food ad libitum during the course of the experiment.

b) Acute toxicity

The acute toxicity of the different tetrachlorophenols and certain related compounds were determined as LD_{50}'s according to Weil (1952). Four groups of four mice C 57 black (weight 20 g) were used for each determination and the compounds were administered orally or intraperitoneally in 40% ethanol or propylene glycol. Some determinations were performed with female gerbils (weight 60 g). The animals were observed for one week. Due to the low toxicity of some of the compounds, tests could not be performed as since they did not dissolve in enough quantities in the solvents used. Tri-CH was not available in quantities enough to allow toxicity determinations.

3. Extraction procedure

1 ml urine was combined with 1 ml concentrated hydrochloric acid

and boiled for 2 hours and after cooling extracted with three aliquots of diethyl ether. The organic phases were combined and carefully evaporated until dryness. The remaining material was derivatized by adding diazoethane and again evaporated until dryness. The residue was redissolved in 0.5 ml hexane and the solution was cleaned by washing with 20% water in methanol, as described by Cranmer and Freal (1970). For the quantitative determination 100 µg · ml^{-1} of an internal standard (see below) was added before boiling and extraction.

4. Gas chromatography - mass spectrometry

Gas chromatography - mass spectrometry was performed on a LKB model 9000 gas chromatograph - mass spectrometer (LKB-Produkter, Bromma, Sweden). The separations were made on silanized glass columns packed with 3% OV-17 on Chromosorb W or 3% SE-52 on Gas Chrom Q and maintained at a temperature of 150°C - 250°C depending on the length of the columns (1.2 - 3.2 m x 3 mm i.d.).

The flow rate of helium carrier gas was 25 ml/min. The ionizing potential and trap current were 70 eV and 60 µA, respectively. The temperature of the flash heater was 210°C and the ion source was kept at 250°C.

For the recordings of mass spectra and the mass fragmentographic analysis the instrument was run under computer control as described by Holmes et al. (1971) and Elkin et al. (1973), respectively. For the mass fragmentographic analysis the ionizing potential was kept at 50 eV and the mass numbers were chosen as described in the respective figures.

Quantitative determination of urinary excretion products were performed with mass fragmentography. A series of standard solutions were prepared by adding known amounts of tetrachlorophenols, tetra-CH and tri-CH to urine collected from unexposed rats and treated in the same way as described for the urinary specimens.

2,3,4,5-tetra-CP was used as internal standard for experiments with 2,3,5,6- and 2,3,4,6-tetra-CP and 2,3,4,6-tetra-CP was used as internal standard for 2,3,4,5-tetra-CP.

RESULTS

1. Purification

The result of the ion exchange column purification of 2,3,4,6-tetra-CP is demonstrated in Fig. 1. The recovery was 60%. A level of about 20% PCP is indicated in the technical product. The detection limit under the conditions used in this case was in the magnitude of 5 ng PCP which indicates that the purified compound thus contains less than 0.1% PCP.

Due to lack of reference compounds the amount of lower chlorinated pyrocatechols in tetrachloropyrocatechol could not be determined but the purification resulted in a 90% reduction of the impurities and they could only be demonstrated close to the detection limit under the conditions mentioned above.

2. Identification of metabolites

The retention times for the different chlorinated compounds discussed are given in Table 1. Derivatives were prepared with diazoethane or with N,O-bis (trimethylsilyl) acetamide, respectively.

The mass spectrometric data for the compounds are summarized in Table 2 which gives the m/e for the base peak, the molecular ion (M+) and the five most intense ions at m/e >100.

The excretion products given in Table 3 have been identified by gas chromatography - mass spectrometry in the urine after administration of the different tetrachlorophenols.

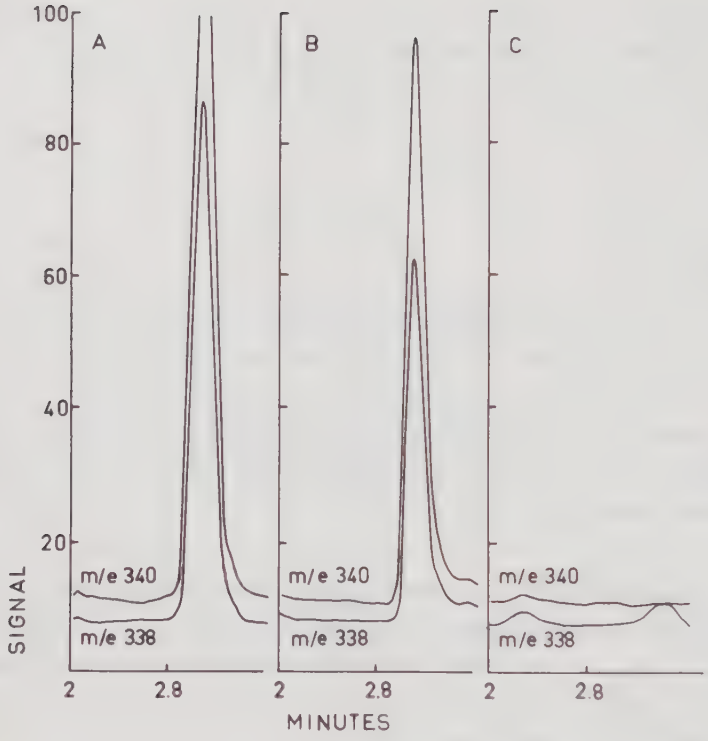

Fig. 1. The mass spectrometer was focussed on m/e 340 and m/e 338 for the detection of PCP. Two channels were focussed on m/e 392 to record tetra-CH and were not used in this case. GC temperature was 200°C and the helium flow rate 25 ml/min. Column used was all glass, 1.6 m, 3 mm i.d., packed with 3% SE-52 on Gas Chrom Q.

A shows the mass fragmentogram after injection of 0.3 µg pentachlorophenol. The recording shows overflow in the channel recording m/e 340 and only m/e 338 was used for the calculations.
B shows the mass fragmentogram after injection of 1 µg of 2,3,4,6-tetra-CP starting material indicating a PCP level of about 20%.
C shows the mass fragmentogram after injection of 5 µg of the purified 2,3,4,6-tetra-CP.

Table 1. Retention times in minutes on 3% SE-52, Gas Chrom Q, silanized glass column 1.6 m, i.d. 3 mm, 150°C, helium flow rate 25 ml/min.

Compound	Ethyl derivative	TMS derivative
2,3,5,6-tetrachlorophenol	1.21	1.61
2,3,4,5-tetrachlorophenol	1.86	1.83
2,3,4,6-tetrachlorophenol	1.23	1.68
Trichlorohydroquinone	2.11	2.73
Tetrachlorohydroquinone	3.06	5.16
Tetrachloropyrocatechol	2.86	4.54

Table 3. Compounds identified in the excreted urine after administration of different isomers of tetrachlorophenol to rats (10 mg/kg, intraperitoneally).

	Excretion products		
Chlorophenol administered	unchanged parent compound	tetra-CH	tri-CH
2,3,5,6-tetra-CP	+	+	-
2,3,4,5-tetra-CP	+	-	(+)
2,3,4,6-tetra-CP	+	-	(+)

Table 2. Mass spectrometric data for reference compounds. The intensity of the five major peaks are given in parenthesis as the percentage of the most intense peak at m/e >100.

Compound	TMS-derivatives			Ethyl-derivatives		
	Base peak m/e	M+ m/e	Five major peaks m/e 100	Base peak m/e	M+ m/e	Five major peaks m/e 100
2,3,5,6-tetra-chlorophenol	93	302	289(100), 287(76), 291(50), 304(13), 302(6)	232	258	232(100), 230(78), 234(48), 131(10), 260(8)
2,3,4,5-tetra-chlorophenol	93	302	289(100), 287(75), 291(49), 304(13), 302(9)	232	258	232(100), 230(78), 234(48), 131(10), 260(8)
2,3,4,6-tetra-chlorophenol	289	302	289(100), 287(75), 291(50), 304(10), 302(7)	232	258	232(100), 230(78), 234(48), 131(10), 260(8)
Tetrachloro-p-hydro-quinone	93	390	392(100), 377(93), 390(73), 270(73), 268(72)	248	302	248(100), 246(78), 250(47), 213(8), 304(6)
Trichloro-p-hydro-quinone	93	356	358(100), 306(97), 356(94), 233(74), 308(69)	212	268	212(100), 214(97), 216(16), 213(13), 268(8)
Tetrachloro-pyrocatechol	73	390	392(100), 390(38), 394(25), 304(15), 302(10)	248	302	248(100), 246(76), 250(24), 247(9), 304(7)

Tetra-CH is a major metabolite of 2,3,5,6-tetra-CP constituting about 35% of the in the urine excreted material. 2,3,4,5- and 2,3,4,6-tetra-CP are excreted essentially unchanged in urine. Tri-CH, however, was identified as a minor metabolite of the latter two compounds but occurred in quantitatively insignificant amounts.

3. Quantitation

The quantitative analysis of urinary excretion products was based on mass fragmentography. A typical mass fragmentographic run of an ethylated standard extract is shown in Fig. 2. Standard curves for 2,3,4,5-tetra-CP, tetra-CH and tri-CH using 2,3,5,6-tetra-CP as internal standard are given in Fig. 3.

The quantitative excretion of the different tetrachlorophenols are given in Table 4, I-III. Each analytical value is based upon extraction of two independent urine samples. For 2,3,5,6-tetra-CP 3.48 mg of the parent compound and 1.87 mg of the metabolite tetra-CH were recovered during the first 24 hours. The recovered amount of tetra-CH corresponds to about 1.75 mg of 2,3,5,6-tetra-CP. The total recovery was thus 5.28 mg, i.e. an almost complete recovery of the administered dose within the first 24 hours. After 24 hours no measurable concentrations of either parent compound or metabolite could be demonstrated.

For 2,3,4,6-tetra-CP the recovery is in the same region but in 48 hours. 2,3,4,5-tetra-CP shows a somewhat different picture with only 60% recovery within 72 hours.

A certain amount both of the tetrachlorophenols and the metabolites may have appeared in the urine as conjugates. This is not revealed in Table 4 because the urine was boiled with concentrated hydrochloric acid in order to split possible conjugates.

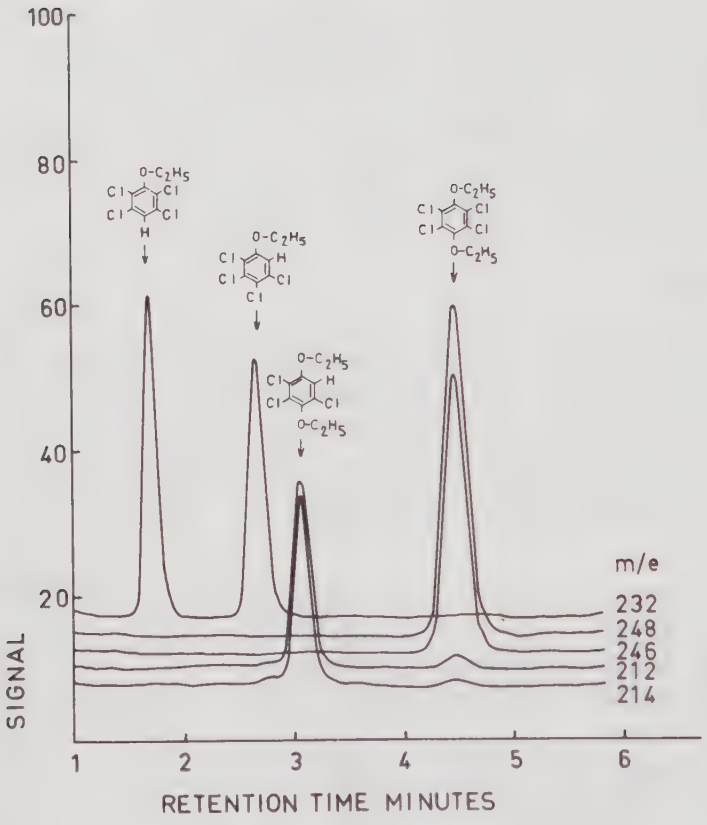

Fig. 2. Mass fragmentogram ethylated standards of tetrachlorophenols and metabolites. m/e 232 was chosen to record tetrachlorophenols, m/e 246 and 248 to record tetra-CH and m/e 212 and 214 to record tri-CH. GC temperature was 140°C and the helium flow rate 25 ml/min. Column used was all glass, 1.6 m, 3 mm i.d., packed with 3% SE-52 on Gas Chrom Q.

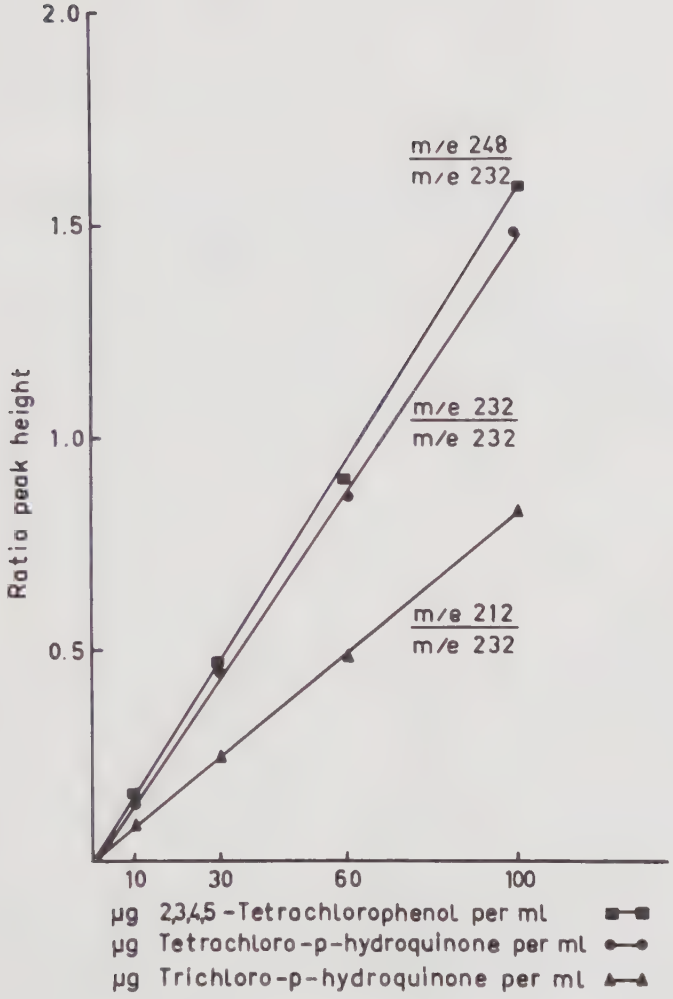

Fig. 3. Standard curves for 2,3,4,5-tetra-CP, tetra-CH and tri-CH using 2,3,5,6-tetra-CP as an internal standard. m/e used are indicated in the figure.

Table 4.

I

2,3,5,6-tetra-CP. Total dose given 5.3 mg.

Collection time, hours	urine volume ml	concentration µg/ml 2,3,5,6-tetra-CP	tetra-CH	total amount excreted, mg 2,3,5,6-tetra-CP	tetra-CH	percent of dose given
0-24	25	139	75	3.48	1.87	98.7
Total amount recovered 5.23 mg (calculated as tetra-CP).						98.7

II

2,3,4,5-tetra-CP. Total dose given 4.9 mg.

Collection time, hours	urine volume ml	concentration µg/ml	total amount excreted, mg	percent of dose given
0-24	20	125	2.50	51.0
24-48	17	20	0.34	6.9
48-72	20	2	0.04	0.8
Total amount recovered 2.88.				58.8

III

2,3,4,6-tetra-CP. Total dose given 4.9 mg.

Collection time, hours	urine volume ml	concentration µg/ml	total amount excreted, mg	percent of dose given
0-24	30	153	4.59	93.7
24-48	27	4	0.11	2.2
48-72	25	0	0	0
Total amount recovered 4.70.				95.9

4. Acute toxicity

The LD_{50} of the different compounds and the respective modes of administration are given in Tables 5-8.

Regardless of way of administration, PCP is more toxic than the respective tetrachlorophenols. In mice the sequence of toxicity of the tetrachlorophenols from high to low is 2,3,5,6-CP> 2,3,4,6-CP>2,3,4,5-CP (Tables 5-7). Given orally to gerbils, 2,3,5,6-CP is the least toxic tetrachlorophenol (Table 8). Tetra-CH and tetra-CPC are less toxic than the corresponding phenols after oral administration (Tables 5 and 6). Tetra-CH, however, is more toxic than the tetrachlorophenols after intraperitoneal administration (Table 5).

DISCUSSION

In earlier studies it has been demonstrated that PCP, after administration to rats or mice, will be partly dechlorinated in para-position leading to the formation of tetra-CH. One could therefore expect that 2,3,5,6-tetra-CP would easily be hydroxylated in the free para-position which also is the case. The other two isomers of tetra-chlorophenol could be expected to undergo declorination in the para-position, in accordance with what happens to PCP resulting in the formation of tri-CH. The latter reaction does not happen to a quantitatively important extent. The occurrence of tri-CH as a urinary metabolite of PCP has been demonstrated by Ahlborg and Thunberg (1977). The type of dechlorination leading to the formation of tri-CH, probably differs from the reaction giving tetra-CH, but constitutes only a minor metabolic pathway.

The tetrachlorophenols are eliminated much faster from the rat than pentachlorophenol. 2,3,5,6-tetra-CP is the only tetrachlorophenol metabolized to a significant degree. This study also demonstrates a difference between the three isomers of tetra-CP

with regard to time of elimination where 2,3,4,5-tetra-CP is excreted considerably slower than the other two isomers.

The toxicity of the tetrachlorophenols is considerably lower than that of the toxicity of PCP. In mice, 2,3,5,6-tetra-CP is the most toxic tetrachlorophenol. This could possibly be explained by its rapid metabolism to tetra-CH, the latter compound displays a toxicity equivalent to PCP when given intraperitoneally. Tetra-CH is easily oxidized to chloranil (tetrachloro-p-benzoquinone) and the low toxicity of tetra-CH, when given orally, may be explained by such an oxidation taking place in the intestine. This explanation is only valid in case the metabolism of 2,3,5,6-tetra-CP is similar in the mouse and the rat, which has been proven to be the case for PCP (Jacobson and Yllner, 1971; Ahlborg et al., 1974) but not yet for 2,3,5,6-tetra-CP.

The tetrachlorophenols used in this study were not analyzed for the occurrence of nonphenolic compounds such as dibenzo-p-dioxins and dibenzofurans. Hexa-, hepta- and octachlorodibenzo-p-dioxins as well as hexa-, hepta- and octachlorodibenzofurans have been shown to occur in commercial grade 2,3,4,6-tetrachlorophenol but could not be shown to influence the embryotoxic and fetotoxic effects of the compound as compared to the effects of purified 2,3,4,6-tetrachlorophenol (Schwetz et al., 1974). On the other hand, a recent study of PCP (Goldstein et al., 1977) has demonstrated that technical PCP, contaminated with chlorinated dibenzodioxins and dibenzofurans, did produce a number of liver changes when administered during 8 months to female rats. Due to the short time of experimentation (<72 hours) it is, however, less likely that a possible occurrence of such contaminants in the tetrachlorophenols studied could have influenced the results of the present study.

Table 5. Acute toxicity of chlorophenols in 40% ethanol administered to female mice C57 black.

Chlorophenol	LD_{50} mg x kg^{-1} orally	95% confidence interval	intraperitoneally	95% confidence interval
Pentachlorophenol	74	(60-90)	32	(22-44)
2,3,5,6-tetrachlorophenol	109	(82-145)	48	(35-67)
2,3,4,6-tetrachlorophenol	131	(107-161)	82	(59-114)
2,3,4,5-tetrachlorophenol	400	(245-656)	97	(73-129)
Tetrachloro-p-hydroquinone	500	(395-631)	35	(24-51)
Tetrachloropyrocatechol	612	($\pm$0)	136	(113-162)

Table 6. Acute toxicity of chlorophenols in 40% ethanol administered to male mice C57 black.

Chlorophenol	LD_{50} mg x kg^{-1} orally	95% confidence interval
pentachlorophenol	36	($\pm$0)
2,3,5,6-tetrachlorophenol	89	(65-122)
2,3,4,6-tetrachlorophenol	163	(123-218)
2,3,4,5-tetrachlorophenol	572	(375-873)
tetrachloro-p-hydroquinone	750	(593-947)
tetrachloropyrocatechol	750	(593-947)

Table 7. Acute toxicity of chlorophenols in propylene glycol administered to female mice C57 black.

Chlorophenol	LD_{50} mg x kg^{-1} orally	95% confidence interval	intraperitoneally	95% confidence interval
pentachlorophenol	150	(113-200)	59	(49-71)
2,3,5,6-tetrachlorophenol	543	(453-664)	109	(82-145)
2,3,4,6-tetrachlorophenol	735	(528-1023)	121	(101-144)
2,3,4,5-tetrachlorophenol	677	(566-810)	133	(98-182)

Table 8. Acute toxicity of chlorophenols in propylene glycol administered to female gerbils.

Chlorophenol	LD_{50} mg x kg^{-1} orally	95% confidence interval
pentachlorophenol	294	($\pm$0)
2,3,5,6-tetrachlorophenol	979	(735-1300)
2,3,4,5-tetrachlorophenol	533	(364-781)
2,3,4,6-tetrachlorophenol	698	(524-930)

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ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Environment Protection Board (7-1/76 and PK-345/76).

LF/ALLF 158 77 068

LiberTryck Stockholm 1977